

UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE QUÍMICA E BIOQUÍMICA



**Probing the synaptic target of a new putative antiepileptic drug:
Modulation of the excitatory transmission in the hippocampus**

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Cátia Isabel Afonso Domingos

Dissertação orientada por:
Prof. Ana Sebastião
Prof. Pedro Lima

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Science is an epic journey of discovery

and “the most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' but 'That's funny...'"

Isaac Asimov

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RESUMO

A complexidade do nosso sistema nervoso permite-nos apreciar momentos de reflexão, arte e imaginação, veiculada ou não pela linguagem. A unidade funcional que o permite é o neurónio, que dispara potenciais de acção seguidos de potenciais sinápticos que no conjunto são considerados a unidade básica de informação. O potencial de acção é um ponto central da regulação do sistema nervoso, que reflete não só a função de canais iónicos e outras estruturas que, ao nível subcelular, o modulam, mas também constitui a base da comunicação entre estruturas cerebrais e determina a coerência ou não de actividade neuronal. Os vários níveis de regulação do potencial de acção e do potencial sináptico interagem entre si e garantem o correcto controlo da excitabilidade. Contudo, dada essa flexível interacção, um pequeno desequilíbrio num dos níveis manifesta-se no sistema inteiro, como é o caso da epilepsia: uma mutação num canal de sódio dependente de voltagem, por exemplo, é suficiente para tornar o indivíduo susceptível a convulsões. Estas caracterizam-se por um desequilíbrio entre o tónus excitatório/inibitório que conduz a actividade neuronal anormal, excessiva e hipersíncrona no cérebro, tornando o indivíduo incapaz de conscientemente controlar o seu corpo por breves momentos, o que resulta frequentemente em fatalidades.

Actualmente, 30% dos pacientes com epilepsia não respondem aos antiepilépticos actuais, ou seja, é fármaco-resistente, o que acarreta 75% do fardo socioeconómico associado a esta doença neurológica. Os mecanismos que estão na base da resistência aos fármacos são partilhados por outras patologias e não estão necessariamente relacionados com os mecanismos de epileptogénese per se. Para ultrapassar esta barreira, uma estratégia a seguir consiste em estudar os mecanismos endógenos anticonvulsivantes do cérebro e potenciá-los, como é o caso da adenosina.

A adenosina é uma molécula ubíqua em todas as células do corpo humano, envolvida em processos básicos de suporte celular (como suporte estrutural nos ácidos nucleicos e suporte funcional na transferência de energia através de ATP). No sistema nervoso, é um importante neuromodulador que apresenta propriedades anticonvulsivantes, principalmente mediadas, mas não restringidas, ao receptor A_1R da adenosina. Estes receptores são os mais abundantes, sendo expressos numa grande variedade de tecidos e órgãos, incluindo o sistema nervoso e o sistema cardiovascular. A activação dos receptores A_1 tem um efeito anticonvulsivante ao diminuir a transmissão sináptica excitatória, diminuindo, assim, a probabilidade de actividade neuronal síncrona e excessiva. Os efeitos da activação dos receptores A_1 manifestam-se pré- e pós sinápticamente. Pré-sinápticamente, a activação dos receptores A_1 acoplados à proteína $G_{i/o}$ resulta na diminuição de cálcio, necessário na libertação de vesículas de neurotransmissor. Assim, observa-se uma libertação de neurotransmissor assíncrona,

responsável por um potencial excitatório pós-sináptico (EPSP) de menor amplitude. Pós-sinápticamente, a activação dos receptores A_1 resulta na activação de canais de potássio, conduzindo à hiperpolarização do potencial de membrana. A redução da transmissão sináptica através dos receptores A_1 deve-se, assim, à menor amplitude do potencial excitatório pós-sináptico, hiperpolarização do potencial de membrana e resistência da membrana diminuindo de modo global a transmissão sináptica.

A utilização de adenosina em modelos de epilepsia fármaco-resistente foi capaz de prevenir convulsões. Contudo, a presença dos receptores A_1 no sistema periférico, nomeadamente no sistema cardiovascular, tornam a administração sistémica da adenosina impraticável, dados os severos efeitos secundários resultantes da depressão da actividade cardíaca. Uma alternativa seria o desenvolvimento de análogos da adenosina que fossem selectivos e de elevada afinidade para os receptores A_1R do sistema nervoso central e não para o sistema periférico.

O fármaco MRS5474, desenvolvido pelo grupo de investigação do Prof. Kenneth Jacobson, apresenta tais características, com um efeito anticonvulsivante num modelo de epilepsia fármaco-resistente (6Hz) quando administrado intraperitonealmente em ratinhos sem os típicos efeitos secundários periféricos observados por um agonista clássico selectivo dos receptores A_1 , CCPA.

O mecanismo de acção do MRS5474 é ainda desconhecido, mas o seu efeito *in vivo* parece apontar para selectividade para o sistema nervoso em vez do sistema periférico. Tal pode ocorrer se o MRS5474 actuar apenas sobre um alvo sináptico que apenas se encontra presente no sistema nervoso central.

Compreender o alvo sináptico e o mecanismo de acção do MRS5474 pode permitir a identificação de um novo alvo para o desenvolvimento de novos fármacos antiepilépticos. Neste trabalho, a hipótese testada foi a de que o MRS5474 diminui a transmissão sináptica excitatória nas células piramidais CA1 do hipocampo através de activação dos receptores A_1 . Esta estrutura é a mais implicada na epilepsia de lobo temporal, o tipo mais comum de epilepsia fármaco-resistente.

De modo a estudar a transmissão sináptica excitatória nas células piramidais CA1, utilizaram-se fatias agudas de hipocampo de ratos wistar wild-type (3-8 semanas). As fibras que estimulam as células piramidais CA1 (Colaterais de Schaffer ou Via Perforante) foram electricamente estimuladas e as correntes pós-sinápticas excitatórias (EPSC) geradas em células piramidais CA1 registadas pela técnica de patch-clamp, no modo whole cell. Para se isolar a componente excitatória da transmissão sináptica, bloqueou-se farmacologicamente os receptores ionotrópicos $GABA_A$ responsáveis pela componente inibitória rápida, adicionando ao meio de perfusão Picrotoxina (50 μM). Após se obter estabilidade no registo da amplitude de EPSP (durante pelo menos 10 minutos) os

fármacos a testar foram adicionados ao meio de perfusão, CCPA (30 nM) ou MRS5474 (120 nM), a concentrações aproximadamente equipotentes para os receptores A₁.

Em primeiro lugar, foi importante testar a activação dos receptores A₁ com um agonista clássico selectivo (CCPA 30 nM), cujos efeitos estão amplamente descritos na literatura para obter um controlo experimental e metodológico. Observaram-se os efeitos esperados da activação dos receptores A₁R: diminuição da amplitude das correntes pós-sinápticas excitatórias em $70 \pm 5,2 \%$ (n=4, p<0.001), diminuição da resistência membranar em $9,9 \pm 1,3\%$ (n=4, p<0.05) e hiperpolarização do potencial de membrana estimado em $6,3 \pm 0,55$ mV (n=3, p<0.001) a partir da medição de corrente injectada para manter o potencial de membrana constante a -70 mV.

Relativamente ao MRS5474, este não alterou significativamente a amplitude dos EPSPs ($128 \pm 12,3\%$, n=8, p>0.05), embora se tenha observado uma elevada tendência para disparo de potencial de acção, contaminando os EPSPs e destabilizando o registo. De modo a isolar-se os EPSPs dos potenciais de acção, utilizou-se um bloqueador de canais de sódio dependentes da voltagem, QX-314 (5 mM), o que reduziu a variabilidade do efeito do MRS5474 sobre os EPSPs mas não o alterou significativamente ($100 \pm 2,40\%$, n=5, p>0.05). Dada a tendência que as células piramidais CA1 apresentaram para disparar potenciais de acção na presença de MRS5474, o seu efeito na excitabilidade intrínseca foi testado através do registo de padrões de disparo. A mesma fatia foi usada para medir padrões de disparo de células piramidais CA1 antes e depois da exposição (>1 hora) ao MRS5474. De facto, a presença de MRS5474 (120 nM) aumentou excitabilidade nas células piramidais CA1, através de despolarização da membrana ($6,1 \pm 1,5$ mV, n=4 Control, n=8 MRS5474, p<0.01), aumento da frequência de disparo de potenciais de acção ($157 \pm 11,8\%$, n=4 Control, n=8 MRS5474, p<0.01) e diminuição da amplitude das correntes rápida (fast) e média (médium) de hiperpolarização da fase de repolarização do potencial de acção ('Afterhyperpolarization' - AHP) fAHP ($42 \pm 8,7\%$, n=4 Control, n=8 MRS5474, p<0.01) e mAHP ($28 \pm 5,7\%$, n=4 Control, n=8 MRS5474, p<0.001) a frequência máxima.

Pode concluir-se que o MRS5474 (120 nM) facilita a excitabilidade das células piramidais CA1 no hipocampo, sem alterar as correntes sinápticas excitatórias, o que por si só não explica a sua acção anticonvulsivante *in vivo*. Pelo contrário, sugere um papel pro-excitatório do fármaco. Contudo, é necessário ter em conta que os resultados obtidos corresponderam às correntes excitatórias de células piramidais CA1 no hipocampo de ratos sem patologia. O efeito de MRS5474 (120 nM) na componente inibitória da transmissão sináptica das células piramidais CA1 assim como o seu efeito em interneurónios, células inibitórias, não foi ainda estudado. Contudo, é fundamental para se poder concluir acerca do mecanismo de acção do MRS5474 como anticonvulsivante, quer

em condições depolarizantes (comuns durante convulsões) quer na excitabilidade de interneurónios.

Palavras-chave: Epilepsia Farmacoresistente, Adenosina, Receptores A₁ para adenosina, MRS5474, CCPA

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ABSTRACT

Activation of adenosinergic system in epileptogenic conditions is an efficient endogenous anticonvulsant mechanism that protects against pharmacoresistant epilepsy. However its systemic administration also triggers unacceptable side effects, mainly cardiovascular, that prevent its use as an antiepileptic drug (AED).

A new adenosine derivative MRS5474 was shown to protect against elicited seizures *in vivo* in a pharmacoresistant epilepsy model without significant side effects, unlike the classical selective A₁R agonist CCPA.

In this work, we aimed at determining the synaptic target of MRS5474 which may help shift the search for new AED towards a novel target. According to binding and adenylate cyclase activity studies, MRS5474 has high A₁R affinity and full agonism with moderate selectivity against other adenosine receptors (e.g. A₃R). We thus hypothesized that MRS5474 decreases excitatory synaptic transmission in CA1 pyramidal cells of the rat hippocampus through A₁R activation.

Acute hippocampal slices from wild-type Wistar rats (3–8 weeks) were used to record excitatory postsynaptic currents (EPSCs) and action potentials (AP) (whole-cell patch clamp) from pyramidal neurons of the CA1 area. Upon electrical stimulation of Schaffer Collaterals, EPSCs were recorded from pyramidal cells under voltage clamp at a V_h of -70 mV. To study MRS5474 (120 nM) effect on excitability properties of the studied cells, action potentials were measured under current clamp after at least 1h exposition to MRS5474. The effect of MRS5474 (120 nM) was compared with that of a standard A₁R agonist, CCPA (30 nM), at an equipotent concentration for the A₁R.

The results show that CCPA (30 nM) had a typical A₁R activation effects: inhibition of excitatory synaptic transmission by decreased EPSC amplitude and membrane potential hyperpolarization, both effects being prevented in the presence of an A₁R antagonist (DPCPX, 100 nM). Unlike CCPA, MRS5474 (120 nM) did not behave as a classical A₁R agonist, since it had no inhibitory effect on excitatory synaptic transmission and even presented a pro-excitatory effect on CA1 pyramidal cell intrinsic excitability properties: resting membrane potential depolarization, increased firing frequency and altered afterhyperpolarization (AHP) current components (decreased fast and medium AHP and increased slow AHP).

So far, a pro-excitatory role of MRS5474 on excitatory synaptic transmission on CA1 pyramidal cells of healthy rat hippocampus is not able to explain the MRS5474 anticonvulsant properties *in vivo* in a model of pharmacoresistant epilepsy (6 Hz). If the increased intrinsic excitability caused by MRS5474 on CA1 pyramidal cells is also observed at a slight greater extent in interneurons, then the global net effect of MRS5474 would be inhibitory. This hypothesis could explain not only its anticonvulsant properties

but also the lack of observable side effects. Further studies evaluating the influence of this drug under more depolarizing conditions (often occurring during seizures) and in the excitability of inhibitory neurons are required to understand the antiepileptic mechanism operated by this drug. Being able to bypass the negative implications of using the adenosinergic system and take advantage of its powerful anticonvulsivant properties is a promising track to fight pharmacoresistant epilepsy.

Abbreviations

A₁R, A_{2A}R, A_{2B}R, A₃R - Adenosine A₁, A_{2A}, A_{2B} and A₃ receptor
aCSF – artificial Cerebrospinal fluid
AED – Antiepileptic drug
AHP - AfterHyperPolarization
AP – Action Potential
AR – Adenosine Receptor
CA1 - Cornu Ammonis area 1
CA3 - Cornu Ammonis area 3
CCPA - 2-Chloro-N⁶-cyclopentyladenosine (selective A₁R agonist)
CNS - Central Nervous System
DG - Dentate Gyrus
DPCPX - 8-Cyclopentyl-1,3-dipropylxanthine (selective A₁R antagonist)
EC - Entorhinal cortex
EPSC – Excitatory Post-Synaptic Current
ER - Endoplasmatic Reticulum
fEPSC – field Excitatory Post-Synaptic Current
GABA - Gamma-AminoButyric Acid
GPCRs - G Protein-Coupled Receptors
HS – Hippocampal Sclerosis
IPSC – Inhibitory Post-Synaptic Current
K_{Ca} – Calcium-activated potassium channels
K_{ir} –Inward rectifier Potassium channels
K_v – Voltage-gated Potassium channels
MRS5474 – 2-Chloro-N⁶-dicyclopropylmethyl-4'-truncated (N)-methanocarba-adenosine
PKA – Protein Kinase A
PLC – Phospholipase C
PRE – Pharmaco-Resistant Epilepsy
PTX – Picrotoxin (GABA_AR antagonist)
QX-314 – Voltage-gated Na⁺ channel blocker
RMP –Resting Membrane Potential
TLE – Temporal Lobe epilepsy
VGCC - Voltage-Gated Calcium channels
V_m – Membrane Potential

INTRODUCTION & AIMS

1. INTRODUCTION

In 400 BC, Hippocrates stated that “(...) All the (...) most difficult [diseases] to be understood by the inexperienced, fall upon the brain.” (Hippocrates, in *On the Sacred Disease*, 400 BC). In 2016, the complexity of our brains continues to inspire neuroscientists to work on a still unresolved question:

“How can a three-pound mass of jelly that you can hold in your palm imagine angels, contemplate the meaning of infinity, and even question its own place in the cosmos? (Ramachandran, 2010).

Even though we are still unable to answer such complex questions, the basic principles of brain functioning have been unraveled. Understanding these basic principles will be necessary to grasp the aim of this work: unraveling the mechanism of action of a new putative antiepileptic drug.

1.1. Neuronal Communication

Using the improved Golgi silver impregnation method, Santiago Ramón y Cajal identified neurons as the functional unit of the nervous system (Ramón y Cajal, 1911). Being individualized units, neuronal communication relies upon signals of different nature, electrical and chemical, so that the information can be carried out within the neuron and between neurons, respectively.

Within the neuron, the information is generated and propagated by an electrical signal known as the action potential (Gasser and Erlanger, 1922). This flow of information is communicated to the next neuron, which is usually in close but not physical contact, through a synapse (Sherrington, 1897). In this structure, the electrical signal is converted to a chemical signal that induces a synaptic potential, traveling along the dendrites to the soma of the post-synaptic neuron. If sufficient, it will trigger the generation of an all-or-none action potential (Adrian, 1914) in the post-synaptic neuron and the information can be carried out in an extremely fast fashion, in the order of milliseconds (ms).

From this simple and brief explanation of neuronal communication, two concepts gain special relevance: the action potential and the synaptic transmission.

1.1.1. The action potential

The action potential is believed to be a basic information unit used by the brain. The electrical excitability that allows a neuron to fire an action potential is based on the presence of proteins embedded in the cellular membrane that are electrically active (Figure 1.). These include two main groups: 1. ion channels that allow electrically charged ions to flow across the membrane and 2. ion pumps that actively transport ions

across the membrane. Ion channels can be divided into three main categories: A. voltage-insensitive, B. voltage-gated or C. ligand-gated. This distinction is based upon its mechanism of action: voltage-insensitive channels (A) are always open while gated channels open upon a signal, being it a difference in membrane voltage (B) or the presence of a molecule (C) which leads to a conformational change, allowing ions to cross the membrane. Ion pumps are responsible for maintaining a negative potential between the intra and extracellular space, the resting membrane potential (RMP), which is primarily generated by voltage-insensitive channels permeable mainly to K^+ . When the membrane, typically at -70 mV, depolarizes gradually due to chemically-activated ion channels to a specific membrane potential, called AP threshold, voltage-gated ion channels are activated and these are the basis for action potential generation.

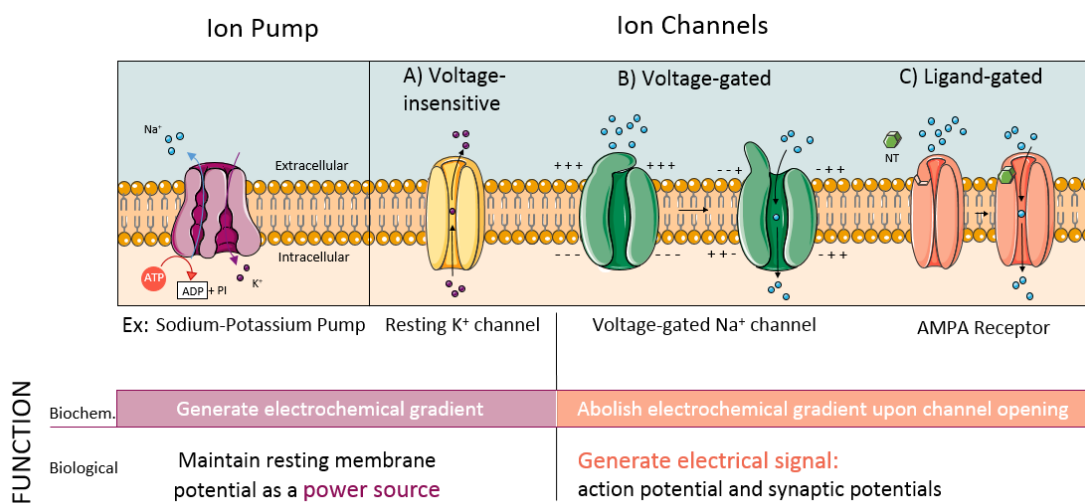


Figure 1. Biomolecular basis for the electrical excitability of neurons.

The resting membrane potential results from an electrochemical gradient maintained by the Na^+/K^+ pump, at energy cost (ATP breakdown), and voltage-insensitive ion channels, mainly K^+ channels. This electrochemical gradient is fundamental as a power source that allows the generation of action and synaptic potentials. This is accomplished by transient electrochemical gradient collapse achieved when ion channels open, upon ligand or voltage sensing, or both. Legend: NT: neurotransmitter.

1.1.2. Phases of the action potential

Hodgkin and Huxley were the first to describe the mechanism of action potential propagation and how several families of voltage-gated ion channels are the basis for the different phases of the action potential, such as sodium, potassium and calcium channels (Hodgkin and Huxely, 1952a-e).

Before briefly analyzing the phases of the action potential, it is fundamental to understand the types of potentials neurons generate. A “potential” results from a difference in electrical charges; neurons exhibit three types of potentials – resting membrane potential, synaptic potential and action potential. The resting membrane

potential (RMP) results from the difference in the electrical charges across the membrane, the intracellular compartment being negative (around -70 mV) and implies a constant energy effort of ion pumps. The synaptic potential occurs at synapses, where the released neurotransmitters from the pre-synaptic neuron bind to ligand-activated ion channels, inducing a local synaptic potential that travels along the dendrite to the post-synaptic neuron soma. This synaptic potential may be excitatory (if it causes membrane potential depolarization) or inhibitory (causes membrane potential hyperpolarization). These potentials are designated by Excitatory Post-Synaptic Potential (EPSP) or Inhibitory Post-Synaptic Potential (IPSP), respectively. If the summation of synaptic potentials depolarizes the membrane potential to its threshold value, an action potential is triggered and the information is carried from one neuron to the next.

The action potential is a transient event in which the membrane potential rapidly rises and falls, in a scale of milliseconds (ms), typically described as in Figure 2. The phases of the neuronal action potential are indicated along with the channels involved in each step. Three main phases can be distinguished: 1. Depolarization, 2. Repolarization and 3. Afterhyperpolarization (AHP).

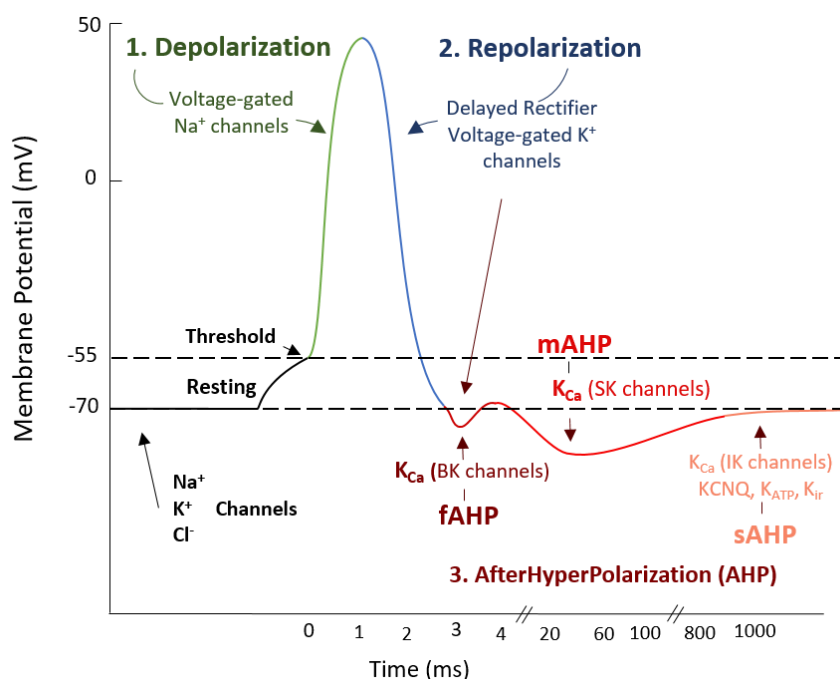


Figure 2. Neuronal action potential. Phases of action potential are indicated along with the channels involved in each step. Three main phases can be distinguished: 1. Depolarization, 2. Repolarization and 3. Afterhyperpolarization, subdivided in fast Afterhyperpolarization (fAHP), medium Afterhyperpolarization (mAHP) and slow Afterhyperpolarization (sAHP). Voltage-gated sodium channels are mainly responsible for the depolarization while potassium channels are mainly responsible for the Repolarization and Afterhyperpolarization. Potassium channels are comprised of three main families: K_V – voltage-gated K^+ channels, K_{Ca} – Ca^{2+} -activated K^+ channels and K_{ir} – Inward Rectifier K^+ channels. Calcium conductance property distinguishes

K_{Ca} channels in BK (Big Ca²⁺ conductance), SK (Small Ca²⁺ conductance) and IK (Intermediate Ca²⁺ conductance). (Based on Goodman, 2008).

1. Depolarization:

Upon sufficient membrane depolarization, closed voltage-gated Na⁺ channels open, increasing permeability to Na⁺, and sodium ions flow inward toward its electrochemical equilibrium potential ($E_{Na} \sim +55$ mV). This causes the depolarization or rising phase of the action potential. Voltage-gated Na⁺ channels are both fast activating and fast inactivating, thus close quickly (at about + 30 mV), before E_{Na} is reached.

2. Repolarization:

Voltage-gated K⁺ channels, which take slightly longer than voltage-gated Na⁺ channels, begin to open and allow K⁺ to move out of the cell, toward its electrochemical equilibrium potential ($E_K \sim -90$ mV). This causes the repolarization or falling phase of the action potential and reestablishes the resting membrane potential.

3. Afterhyperpolarization:

Voltage-gated K⁺ channels and other K⁺ channels remain open and the membrane potential undershoots the resting membrane potential, approaching the electrochemical equilibrium potential for K⁺ ($E_K \sim -90$ mV). This phase is called Afterhyperpolarization (AHP) or refractory period. It is constituted of 4 phases that can be described upon their temporal dynamics and underlying currents (Table 1):

3.1. fast AHP (fAHP)

Lasting 1 to 2 ms, the fAHP follows single action potentials and is carried by 1. Voltage-gated K⁺ channels, opened during the repolarizing phase that remain open, and 2. calcium and voltage-gated BK channels (Storm 1987, Sah and Faber, 2002). These channels belong to a family of calcium-activated potassium channels that may be distinguished based on their potassium conductance:

- BK (Big K⁺ conductance channels)
- IK (Intermediate K⁺ conductance channels)
- SK (Small K⁺ conductance channels)

BK channels are activated by voltage changes and micromolar Ca²⁺ concentrations (due to N-type Ca²⁺ channels). Co-localization of N-type Ca²⁺ channels and BK channels was observed in many neuron types, including CA1 pyramidal cells (Marrion and Tavalin, 1998). Calcium sensing by BK channels is direct, due to the presence of a cytoplasmic C-terminal domain (CTD) (Wu et al. 2010).

3.2. After-DePolarization (ADP)

Lasting 3-4 ms, the ADP follows single action potentials and is mediated, in part, by a Ca^{2+} tail current mediated by R-type Ca^{2+} channels (Metz *et al.*, 2005) and may also have a contribution from persistent Na^+ current (Yue *et al.*, 2005).

3.3. slow post-burst AHP

This component may be divided into two components: the medium AHP (mAHP) and slow AHP (sAHP). After a single action potential, it is only possible to measure mAHP, whereas after a train of action potentials it is possible to identify mAHP and sAHP, which lasts more than hundreds of ms up to seconds.

3.3.1. medium AHP (mAHP)

Lasting 20 - 100 ms, the mAHP is carried by apamin-sensitive SK (Small- K^+ conductance) (Sah and Faber, 2002). SK channels are insensitive to voltage and are activated by submicromolar Ca^{2+} concentrations (due to L-type Ca^{2+} channels). Similarly to BK channels, SK channels co-localize, at the pre-synaptic terminal, with L-type Ca^{2+} channels (Marrion and Tavalin, 1998; Bowden *et al.*, 2001), through which Ca^{2+} influx is directly sensed by a calmodulin domain in SK channels (Adelman, 2016). Both of these calcium-activated potassium channels (K_{Ca} channels), SK and BK channels, highlight the importance of submembrane calcium microdomains: Ca^{2+} can reach concentrations up to 200 to 300 micromolar and this information can be specifically sensed by determined channels without affecting other calcium signaling pathways (Rizzuto and Pozzan, 2006).

3.3.2. slow AHP (sAHP)

The sAHP develops after the firing of a train of action potentials with a time course of hundreds of milliseconds and decays with a time course of seconds. It was first described more than 30 years ago (Hotson and Prince, 1980; Alger and Nicoll, 1980; Schwartzkroin and Stafstrom, 1980) but the underlying channels have only recently been uncovered. Ca^{2+} -activated K^+ channels have long been thought to be in the basis of sAHP (Hotson and Prince, 1980; Alger and Nicoll, 1980; Schwartzkroin and Stafstrom, 1980; Brown and Griffith, 1983; Madison and Nicoll, 1984; Lancaster and Adams, 1986; Schwindt *et al.*, 1992).

The potassium channels underlying sAHP have been recently identified: 1. KCNQ, 2. IK channels, 3. K_{ATP} and 4. K_{ir} .

Unlike fAHP and mAHP, the sAHP depends on diffusible cytosolic calcium sensors (like Hippocalcin and neurocalcin δ (Tzingounis *et al.*, 2007) that sense the intracellular Ca^{2+} . Hippocalcin, for example, upon binding to intracellular Ca^{2+} , exposes a myristoylated group and translates to the plasma membrane, to activate the sAHP potassium channels in the plasma membrane.

Table 1. Summarized properties of AfterHyperPolarization components of Action Potential

Component	fAHP		ADP	mAHP		sAHP		
Developed after:	single AP		single AP	single AP or train of APs (similar properties)		train of APs		
Duration	1-2 ms		3-4 ms	20-100 ms		100 ms - >1 s		
Membrane Polarization	Hyperpolarization		Depolariz.	Hyperpolariz.		Hyperpolarization		
Underlying channels	K _v : Voltage-gated K ⁺ channels	K _{Ca} : Big K ⁺ conductance channels (BK)	Persistent Na ⁺ current Ca ²⁺ tail current	K _{Ca} : Small K ⁺ conductance channels (SK)	K _{Ca} : Intermediate K ⁺ conductance channels (IK)	K _v : KCNQ family	K _{ATP}	
(depends on cell type)								
Specific Blockers		Ibidiertoxin		Apamin Bicuculline-m		TRAM-34	UCL2077	Glibenclamide
Channel properties	Voltage-gated	+	+		-		+	-
	Ca ²⁺ -activated	-	+ (mM)	+	+ (<mM)		+	-
	Conductance		100-250 pS	-	5-20 pS	~40 pS	~2-9 pS	~25 pS
Ca ²⁺ source	Channel	-	N-type Ca ²⁺ channel	R-type Ca ²⁺ channel	L-type Ca ²⁺ channel	Depends on cell type	N-, P- and Q-type Ca ²⁺ channels	-
	Ca ²⁺ sensing by underlying channel	-	Direct Microdomain source (Co-localization)	-	Direct Microdomain source (Co-localization)	Direct Microdomain source -	Indirect Bulk source (Cytosolic diffusible sensors: Hippocalcin and neurocalcin δ)	-
	Specific Blockers	-	ω-Conotoxin GVIA	-	Nifedipine		ω-Agatoxin-IVA (P, Q-type)	-
Function		Shape of AP		Shape and frequency of AP		Prevents over-excitation over repetitive or long-lasting stimulus inputs.		
Affected processes		Learning		Epilepsy Memory Synaptic plasticity Aging				
Characteristics		Remain open after repolarization phase for 1-2 ms		Amplitude increases with number of AP fired Strongly modulated by NT				

The fact that sAHP is activated by cytosolic calcium sensors is consistent with early findings that the sAHP seems to be sensing the bulk calcium in the cytosol and not the calcium inflow in microdomains, as is the case for the fAHP and mAHP. It also reconciles the fact that sAHP current occurs with a 150 ms delay of Ca²⁺ channels activation when no calcium ions are available no more. This is now explained by this transduction system of a calcium sensors. The K⁺ channels that are activated through this process are the 1. KCNQ channel family members (potassium channels, voltage-gated, KQT-like family). They were shown to contribute to sAHP in CA1 and CA3 pyramidal cells (Tzingounis *et al.*, 2008 and 2010) and, later, a specific blocker of sAHP, UCL2077, was shown to block KCNQ channels (Soh and Tzingounis, 2010), further strengthening the idea that KCNQ underlie sAHP. However, even though blockage of Hippocalcin or blockage of KCNQ had significantly reduced the sAHP (by over 50% in CA3 pyramidal cells), other channels need to contribute to sAHP since knockout of specific members of the KCNQ family did not completely abolish the sAHP.

Also, even though Hippocalcin is highly expressed in hippocampal neurons, it is not highly expressed in all parts of the brain that exhibit a sAHP (Villalobos and Andrade, 2010).

Other Ca^{2+} -dependent channels were shown to underlie the sAHP in CA1 pyramidal cells. Belonging to the Ca^{2+} -dependent K channels (SK, BK), 2. IK channels (also named SK4, IKCa, KCa3.1.) underlie the sAHP, reducing temporal summation of EPSPs and mediating spike accommodation (King *et al.*, 2015). Similarly to SK and BK channels, calcium is sensed by IK channels through a direct mechanism: a calmodulin domain in C-terminal. Pharmacological blockade or genetic knockout of these channels completely abolish the first 5-30 ms of sAHP amplitude, increased AP frequency and decreased spike accommodation of a train of APs. This highlights its importance in sAHP in CA1 hippocampal neurons.

However, these channels were only recently found in specific neurons (Wulff *et al.*, 2007: not detected in the CNS; Engbers *et al.*, 2012: detected in cerebellar Purkinje neurons; Turner *et al.*, 2015: detected in rat or mouse neocortex, hippocampus, thalamus, and cerebellum with differential pattern of distribution between cells). This heterogeneous distributions suggests that there are several channels contributing to the sAHP, maybe even different channels in different cell types. K_{ATP} channels, for example, have been suggested to contribute to sAHP in granule cells in the mouse dentate gyrus, since a K_{ATP} blocker, glibenclamide, reduces the amplitude of sAHP in these cells (Tanner *et al.*, 2011).

Common to all neurons, inwardly rectifier K^+ channels (K_{ir} channels) (Isomoto *et al.* 1997) are activated by hyperpolarization, leading to inward fluxes of K^+ that depolarize the axon and bring the membrane potential back toward the resting membrane potential. K_{ir} channels open after ~30 ms following an action potential and reach steady state over 100-200 ms. Thus, they are involved in the last phase of recovery toward resting membrane potential following an action potential or a train of action potentials.

1.1.3. Functional interpretation of action potential and its components in health and disease

The action potential (AP) components, especially the AfterHyperpolarization (AHP), have an important function role at a network level. This results from the central role that the action potential(s) assumes in neuronal communication, connecting all levels of organization.

We may describe the multiple levels of networks in the nervous system in a simplified way consisting in:

- nanocircuits – computation within a neuron, resulting from the underlying biochemical machinery that mediates key neuronal properties;
- microcircuits - few interconnected neurons may form a microcircuit and perform sophisticated tasks such as mediate reflexes, process sensory input and generate locomotion;
- macrocircuits - multiple embedded microcircuits that mediate higher brain functions such as object recognition and cognition.

Even though specific mechanisms of modulation work in each circuit level, modulation also spans all levels and a single change at a nanocircuit level may have reverberant effects at a macrocircuit level in health and disease. This is the case for AHP, which functional role not only regulates neuron electrical excitability but is also associated with higher functions, such as memory and learning, and dysfunctions, such as epilepsy.

Through K^+ channels, the AHP prevents overexcitation of the neuron in response to repetitive or long-lasting stimulus inputs (mAHP and sAHP) (Hille, 2001), determines firing frequency (mAHP) and bursting frequency (sAHP) (Gu *et al.*, 2007) and shapes firing patterns (sAHP), thus modulating nanocircuit activity. The frequency of firing and bursting and the shape of firing patterns assume special roles at a macrocircuit level, in network synchronization and higher functions (and dysfunction). A common feature of AP firing patterns *in vivo* is bursting (Kandel and Spencer, 1961; Ranck, 1973; Fox and Ranck, 1975; Suzuki and Smith, 1985) and its frequency is regulated by sAHP. The sAHP is modulated by activity (amplitude increases with the number of APs fired (Hotson and Prince, 1980; Madison and Nicoll, 1984) and neurotransmitters, allowing them to alter the firing properties of the neuron (Madison and Nicoll, 1982; Andrade and Nicoll 1987; Haas and Konnerth, 1983). It has been shown that sAHP correlates with learning and aging (Disterhoft *et al.*, 2004; Oh *et al.*, 2010) and its malfunction is implicated in epilepsy (McCormick and Contreras, 2001).

Neuronal activity coherence and stability lies in a very tightly regulated interaction between all levels of networks (nano-, micro- and macrocircuits). This stability may be imbalanced by a single mutation in one ion channel, easily leading to a situation of

uncontrolled overexcitation or depression. This is especially important in epilepsy, which can result from disturbance in all levels of organization, from the biochemical level (channelopathies) to the cell level (specific cell type death) to microcircuit level (recurrent excitation), manifested in a macrocircuit level (generalized seizure) (see Table 2 for summarized identified causes of epilepsy).

Table 2. Summarized Functional level abnormalities in Epilepsy

Level	Biological Basis	Examples
Nanocircuit	Ion Channelopathies ⁽¹⁾	Mutations in sodium ⁽²⁾ , calcium ⁽³⁾ , potassium ⁽⁴⁾ and ligand-gated channels
Microcircuit	Abnormal network plasticity and/or Changes in the epileptogenic substrate/network ⁽⁵⁾	<ul style="list-style-type: none"> • Loss of excitatory neurons “driving” inhibitory neurons⁽⁶⁾ • Loss⁽⁷⁾ or mal-development⁽⁸⁾ (migration and maturation) of interneurons • Reactive gliosis (e.g. hippocampal sclerosis ⁽⁹⁾ , cortical dysplasia)
Microcircuit and Macrocircuit	Seizure-induced synaptic reorganization: development of epileptic circuits within and between brain regions ⁽¹⁰⁾ Leading to aberrant neuronal synchronization ⁽¹¹⁾	<ul style="list-style-type: none"> • Excitatory compensatory axonal sprouting in the hippocampus⁽¹²⁾ (Microcircuit) • Limbic network synchronization⁽¹³⁾ (Hippocampus, amygdala, entorhinal cortex, olfactory cortex and the midline thalamic nuclei) (Macrocircuit) • Aberrant oscillatory rhythms⁽¹⁴⁾ (by circuits connecting the cortex and thalamus – Thalamocortical Rhythms) (Macrocircuit)

(1) George, 2004

(2) Meisler *et al.*, 2010

(3) Gambardella and Labate, 2014

(4) D’Adamo *et al.*, 2013

(5) Paz and Huguenard, 2015

(6) Biagini *et al.*, 2005

(7) Liu *et al.*, 2014

(8) Kriegstein, 2005; Powell, 2013

(9) Thom, 2014

(10) Caciagli, 2014

(11) Meisel, 2016

(12) Sutula, 2002

(13) Jefferys *et al.*, 2012

(14) Huguenard, 1999

1.2. Imbalance in neuronal activity: Epilepsy

Epilepsy is one of the most common serious neurological disorder worldwide with no age, racial, social class, national nor geographic boundaries (WHO, 2004). It affects 0.5-2.0% of the global population (Hauser *et al.*, 1998; Picot *et al.*, 2008) and it is estimated that 2.4 million are diagnosed with epilepsy every year (Hirtz *et al.*, 2007).

Nowadays it is clear that perturbed neuronal activity is in the basis of epilepsy, but this is a relatively new concept in the History of mankind. Indeed, epilepsy is an ancient disorder, in the sense that it can be traced as far back as medical records exist to the medical texts of the Assyrians and Babylonians in the 2000 BC. Since then and until recently, epilepsy was seen not as a physical disease but as a spiritual invasion of the body by an evil spirit. For this reason, the physical clinical observation of epilepsy was named seizure (from the Latin *sacire* – to take possession of). Even though Hippocrates, in 400 BC, had been the first to identify the brain as the physical structure affected (in *On the Sacred Disease*, 400 BC), the idea of epilepsy being a spiritual affliction remained unchanged. For this reason, epileptics were treated as lunatics or possessed until the 18th and 19th centuries. In the 19th century, electrical neuronal activity was declared to be the source of such disorder (Fritsch and Hitzig, 1870; Sidiropoulou *et al.*, 2010) but a better understanding of the mechanisms of the disease, the development of effective drugs and neurotechnology for a successful yet incomplete medical therapeutic only emerged progressively throughout the 20th century (for a review see Magiorkinis *et al.*, 2014).

As pointed out in the previous chapter, Epileptogenesis, the term given to the processes that originate epilepsy, encompasses very broad phenomena and it probably differs for the various types of epilepsy (Schuele and Lüders, 2008). For this reason, many authors consider that many “epilepsies” exist, rather than a single one. This reality is also mirrored by the diversity of seizures patients experience, which may be categorized by seizure foci (partial or generalized) and type (simple or complex if it is partial, and in tonic-clonic, absence, myoclonic and atonic, if generalized). Despite the broad epileptogenesis mechanisms, the outcome is the same: seizures - an imbalance between excitatory/inhibitory tonus that leads to an abnormal, excessive, hypersynchronous neuronal activity in the brain, rendering the individual unable to consciously control its body for briefs moments (Goldberg and Coulter, 2013). It can vary from uncontrolled jerking movement (tonic-clonic seizure) to as subtle as a momentary loss of awareness (absence seizure). Epilepsy is characterized by an enduring predisposition to generate epileptic seizures, though seizures can also occur sporadically in people who do not have epilepsy (for further reading on Epilepsy, see Kandar *et al.*, 2012).

Despite the broad epileptogenesis mechanisms, successful therapeutic strategies employed today counteract the exacerbated neuronal excitability. About two thirds of the epileptic patients are successfully treated with antiepileptic drugs (AEDs) that act mainly by increasing GABAergic system or blocking Na^+ channels, even though other AEDs act upon other targets (see Figure 3).

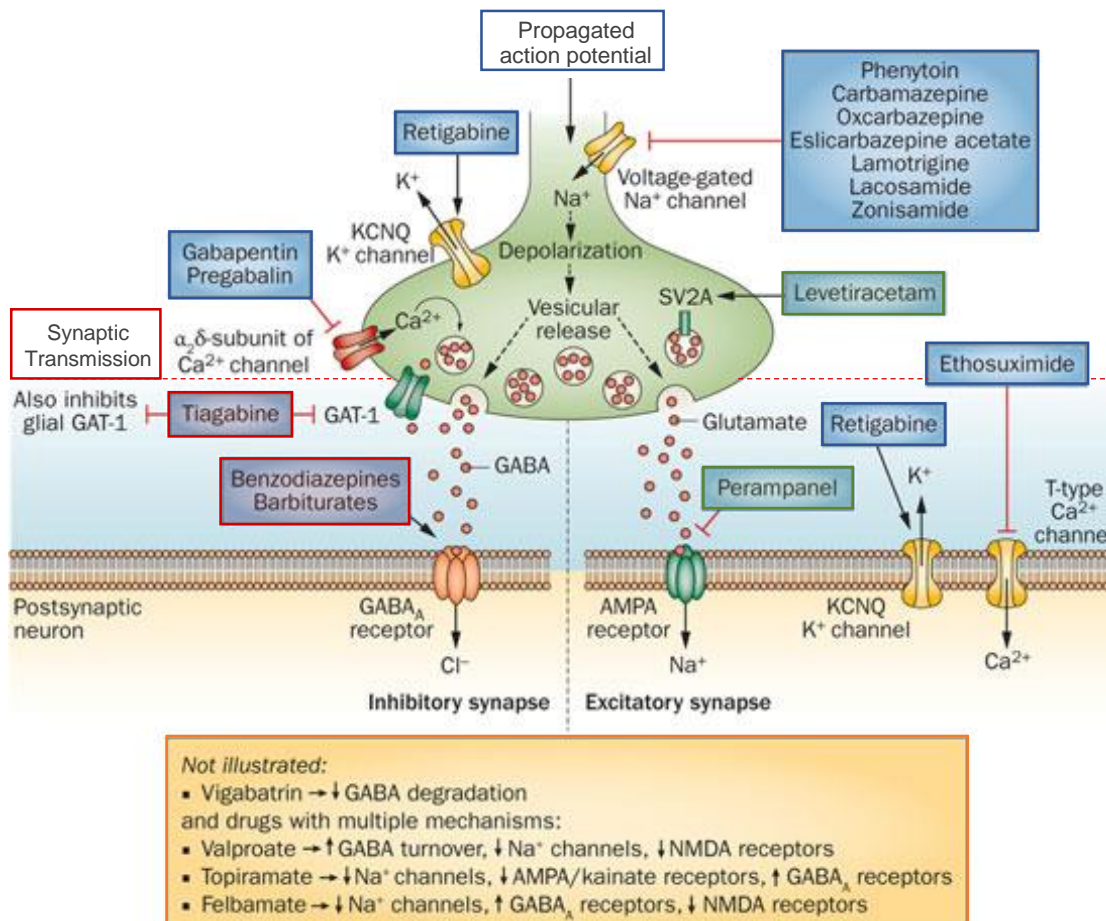


Figure 3. Mechanisms of action of clinically approved AEDs. Neuronal activity is decreased by interfering with action potential generation (through voltage-gated ion channels: Sodium channels blockers, Potassium channels openers and Calcium channel blockers, colored in blue) and by interfering with synaptic transmission, either GABAergic system (lower left panel – inhibitory synapse, colored in red) or Glutamatergic system (lower right panel – excitatory synapse, colored in green). Some AEDs present multiple mechanisms of action, colored in orange. (Adapted from Bialer and White, 2010).

1.2.1. Pharmaco-Resistant Epilepsy

In spite of the availability of different class of anti-epileptics therapies, about one third of the patients is pharmacoresistant (Kwan and Brodie, 2000; Sillanpaa *et al.*, 1998). Despite the discussion around the definition of pharmacoresistance, it is nowadays identified when seizure management is not achieved by a first or a second anticonvulsant medication at daily therapeutic dosage (Kwan *et al.*, 2010).

Pharmaco-Resistant Epilepsy (PRE) poses a serious threat to global human health and wealth since it increases 2 to 10-fold the risk of sudden death when compared to the general population (Chapell *et al.*, 2003) and it represents the major socioeconomic burden for neurological disorders, estimated in over 3 billion \$ in a given year in US (Murray *et al.*, 1996), 75% of which is due to indirect expenses, such as loss of productivity from unemployment, underemployment or lost work time and lost work of relatives and friends, who care for the patient with PRE (Platt and Sperling, 2002). The critical aspect of PRE, aside being a medically, physically and social disabling disorder (Kwan and Brodie, 2002), is the fact that it is progressive, as Hippocrates stated: "It is also curable (...) unless by long lapse of time it be so ingrained as to be more powerful than the remedies that are applied." (in *On The Sacred Disease*, 400 BC). Nowadays, the fact that a minority of patients refractory to currently used drugs may become seizure free with new AEDs (Bazil, 2002) seems to indicate that no absolute intractability exists. Thus, medically intractable seizures may become tractable, once we understand more about the mechanisms underlying intractability and how to prevent or reverse this state.

1.2.2. Biological basis for Pharmaco-Resistant Epilepsy

Pharmacoresistance is not unique to epilepsy: it is now recognized in diverse brain disorders, including depression and schizophrenia (Löscher and Potschka, 2005) and in other diseases affecting the brain, such as human immunodeficiency virus infection and many forms of malignant neoplasias (Siddiqui *et al.*, 2003). As stated by Alexopoulos, the failure of multiple AEDs on treating PRE patients points towards a dysfunction not specific for a single AED target, but to a higher dysfunction that interferes with all AEDs mechanisms of action in general. Hence, the mechanisms of PRE are not necessarily the same as those underlying the epileptogenic process per se (Granata *et al.*, 2009).

Different scenarios or patterns of multidrug resistance exist in epilepsy. In many patients with medically intractable epilepsy, epilepsy is refractory from the onset (*de novo* resistance), suggesting that intrinsic (constitutive) factors are involved in intractability (Arroyo *et al.*, 2002). However, in other patients, drug resistance may arise during the course of epilepsy after an initially positive response, suggesting that epilepsy-related, acquired changes affecting AED efficacy or progression of the disease are involved in intractability (Progressive drug-resistance)(Arroyo *et al.*, 2002). In some cases, drug resistance to a specific AED arises after an initially positive response, but disappears with a new AED trial (waxing and waning resistance). Thus, any hypothesis of multidrug resistance has to deal with these and possibly other patterns in the occurrence and course of intractability in epilepsy.

Mechanisms in the origin of PRE

Pharmacoresistance mechanisms still remain to be fully elucidated. The main hypotheses that – at least in part – explain pharmacoresistance include the transporter hypothesis, the target hypothesis, the network hypothesis, the gene variant hypothesis and the intrinsic severity hypothesis (see Table 3.) (Remy and Beck, 2006; Löscher *et al.*, 2013; Schmidt and Löscher, 2009). However, none of these hypothesis stands alone and is able to convincingly explain how drug resistance arises in human epilepsy (Schmidt and Löscher, 2009). Moreover, they are not mutually exclusive and may be either constitutive or acquired during the course of the disease (Pati and Alexopoulos, 2010).

Table 3. Hypothesis in the basis of Pharmaco-Resistant Epilepsy

Hypothesis	Mechanism	Clinical proof
Transporter	Suggests that inadequate penetration of AEDs across the blood–brain barrier (caused by increased expression of efflux transporters such as P-glycoprotein and multi-drug resistance proteins (MRPs) leads to insufficient drug levels in epileptogenic brain tissue. (Löscher and Potschka, 2005; Schmidt and Löscher, 2005);	Inhibiting the efflux transporter P-glycoprotein counteracted resistance to AEDs in a rat model of pharmacoresistant temporal lobe epilepsy (Brandt <i>et al.</i> , 2006)
Altered Target	Suggests that acquired alterations to the structure and/or functionality of target ion channels and neurotransmitter receptors lead to insufficient pharmacodynamic activity of AEDs in the brain;	A reduced sensitivity of major targets for many of the clinically established AEDs (Rogawski and Löscher, 2004), such as the voltage-gated sodium channel and the GABA _A (γ-aminobutyric acid type A) receptor, has been suggested to have a role in AED-resistant chronic human and experimental epilepsy (Schmidt and Löscher, 2005; Remy and Beck, 2006)
Network	Proposes that structural brain alterations and/or network changes (for example, hippocampal sclerosis) are involved in resistance to AEDs;	Surgical resection of the altered network counteracts AED resistance and may even cure epilepsy (Wiebe and Jette, 2012)
Gene variant	Suggests that there is an inherent resistance that is governed by genetic variants of proteins that are involved in the pharmacokinetics and pharmacodynamics of AED activity (Löscher <i>et al.</i> 2009);	
Intrinsic Severity	Suggests that an increased disease severity leads to drug intractability (Rogawski, 2013).	

The most common form of pharmaco-resistant epilepsy is Temporal Lobe epilepsy (TLE) and is usually associated with hippocampal sclerosis (HS) (Thom, 2014). For several decades, HS has been considered the source of the electrical events that cause spontaneous seizures (Falconer, 1974). However, HS is found in approximately 40-65% of patients who undergo surgery for TLE (de Lanerolle *et al.*, 2003), and whether hippocampal sclerosis is the cause or the consequence of repeated seizures is still a matter of debate (Jefferys, 1999; Boison, 2008).

Hippocampal sclerosis includes three main features (for a review see Thom, 2014):

1. Selective loss of neuronal cells (both excitatory and inhibitory)
2. Axonal sprouting
3. Gliosis

The selective loss of inhibitory neurons (interneurons) as long been regarded as a primary cause for imbalanced neuronal activity, which applies to both pharmacoresistant and pharmacosensitive epilepsies. As mentioned before, inhibitory neurons are responsible to control excitability and regulate neuronal communication. They comprise a highly heterogeneous class of neurons, with more than 20 types of interneurons in the hippocampus with different morphology, molecular expression, axon projection and discharge pattern (Liu *et al.*, 2014). Their heterogeneity endows them with different properties suited to perform different microcircuit functions (see Figure 4.) present in the hippocampus.

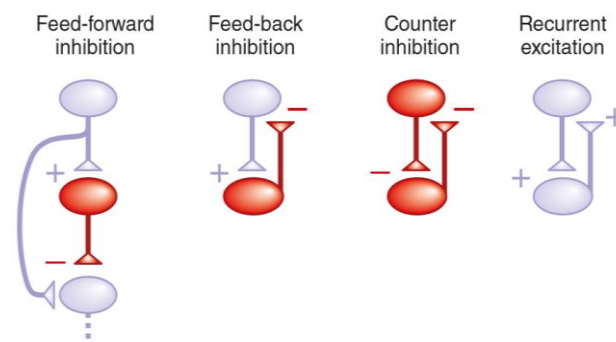


Figure 4. Microcircuit motifs whose dysfunction have been identified in epilepsy. Feed-forward inhibition: excitatory inputs from remote brains regions recruit local inhibitory networks that control the strength of the efferent signal. Feed-back inhibition: local activation of inhibitory neurons controls local recurrent excitatory activity. Counter-inhibition: local connections between inhibitory neurons shape network-inhibitory output. Recurrent excitation: major mode of connectivity in cortical networks. Purple and red represent excitatory glutamatergic and inhibitory GABAergic neurons, respectively. From Paz and Huguenard, 2015.

Selective loss of interneurons leads to microcircuit network destabilization and hyperexcitability since this operations (feed-back inhibition and counter inhibition, for example) are disrupted. Upon loss of excitatory and inhibitory neurons, occurs an attempt of the system to recover the lost wiring circuits, by establishing new synapses

with survival neurons. However, this process of axonal sprouting may be unsuccessful leading to an abnormal network reorganization which is seen as a cause for hyperexcitability in HS.

Gliosis is another hallmark of HS. It refers to an abnormal morphology and function of glia cells (astrocytes, microglia and oligodendrocytes) in response to damage. Astrocytes have been recently shown to play an important role in epilepsy dysfunction (Binder and Steinhäuser, 2006; Coulter and Steinhäuser, 2015). They are involved in many functions that were observed to be dysfunctional in epilepsy, namely Blood-Brain Barrier maintenance, homeostasis of extracellular ions, mainly K^+ , adenosinergic system dysfunction, synaptic communication (tripartite synapse) and electrical coupling in astrocyte-astrocyte communication (see Table 4.) (Coulter and Steinhäuser, 2015). More specifically, investigation of specimens from patients with pharmacoresistant temporal lobe epilepsy and epilepsy models revealed alterations in expression, localization and function of astroglial K^+ and water channels, entailing impaired K^+ buffering. Moreover, malfunction of glutamate transporters and the astrocytic glutamate-converting enzyme, glutamine synthetase, as observed in epileptic tissue, suggested that astrocyte dysfunction is causative of hyperexcitation, seizure spread and neurotoxicity (Coulter and Steinhäuser, 2015). Even though it is not clear yet whether gliosis is a cause or consequence of epileptic activity, it is in a special position to explain pharmacoresistance mechanism hypothesis, common to different patients.

Table 4. Astrocyte (Dys)Function in Epilepsy

Physiological function	Epilepsy	Example
Trafficking of substances from BBB to neurons	Compromised Blood–brain barrier (BBB) ⁽¹⁾	Long-lasting breakdown of the (BBB) leads to astrocyte GFAP upregulation, followed by development of an epileptic focus ⁽²⁾
Homeostasis of extracellular ions, principally potassium	Activity-dependent shifts in ion concentrations contributes to ictogenesis ⁽³⁾	Tetanic stimulation of afferents as well as chemically induced seizure activity increases K^+ , compromising the resting membrane potential of neurons and cotransport of other ions ⁽⁴⁾
Homeostasis of extracellular adenosine concentration by adenosine kinase ADK	Dysfunction in adenosinergic system has been associated with epilepsy ⁽⁵⁾	Mice overexpression of ADK are prone to seizures and exhibit cognitive impairments seen in epilepsy ⁽⁵⁾ Decreased A_1R is associated with epilepsy ⁽⁶⁾
Astrocyte-Astrocyte communication	Disruption of Astrocyte-Astrocyte communication	Electrical coupling between astrocytes is disrupted in TLE patients ⁽⁷⁾

(1) Seiffert *et al.*, 2004; Ivens *et al.*, 2007; Tomkins *et al.*, 2007

(5) Boison, 2008

(2) David *et al.*, 2009

(6) Boison, 2012b

(3) Fisher *et al.*, 1976; Staley *et al.*, 1995; Raimondo *et al.*, 2013

(7) Steinhäuser *et al.*, 2012

(4) Ransom *et al.*, 2000

1.2.3. Treatment for Pharmaco-Resistant Epilepsy

In Pharmaco-Resistant Epilepsy, AEDs are not able to render the patient seizure-free. For this reason, other pharmacological and non-pharmacological treatments are applied (see Table 5) (Sharma *et al.*, 2015).

From the non-pharmacological treatments, surgical procedures are the most widely applied whenever possible and have been successful in 65% of the cases (Wiebe and Jette, 2012). However, it is often not possible to proceed that way and other pharmacological and non-pharmacological procedures are applied.

From the pharmacological treatments, one of them is considered to be an endogenous inhibitory mechanism that is triggered by elevated network activity: adenosine. Studying it is a promising strategy for developing anti-epileptic strategies (Löscher and Kohling, 2010).

Table 5. Main Treatments for Pharmaco-Resistant Epilepsy

	Treatment	Examples
Pharmacological	Add-on drug to treat pharmaco-resistance mechanism (e.g. the transporter hypothesis)	<ul style="list-style-type: none"> • Verapamil, a P-gp inhibitor that avoids AED transport efflux, in conjunction with an AED (Carbamazepine)
	Neuromodulators with protective endogenous properties	<ul style="list-style-type: none"> • Neuropeptide Y • Adenosine
	Others molecules presenting antiexcitotoxicity, anticonvulsivant and neuroprotective properties	<ul style="list-style-type: none"> • Atorvastatin • Thyrotropin-releasing hormone
Non-Pharmacological	Surgical procedures: <ul style="list-style-type: none"> • Seizure foci removal • or Disconnection 	<ul style="list-style-type: none"> • Anteromedial temporal lobectomy • Focal neocortical resection • Lesional resection • Hemispherectomy • Corpus callosotomy • Multiple subpial transections
	Neurostimulation	<ul style="list-style-type: none"> • Vagal nerve stimulation • Responsive neurostimulation • Thalamic stimulation • Transcranial stimulation
	Dietary Therapies Promote production of endogenous anticonvulsivant molecules, as adenosine and GABA, since ketonebodies processing is different between brain and liver of epileptic patients	<ul style="list-style-type: none"> • Classic ketogenic diet • Medium chain triglyceride • Modified Atkins diet • Low glycemic index treatment
	Focal cooling	Rapid cooling of the brain reduces epileptiform activity and may also terminate focal seizures
	Herbal remedies	Cannabinoids

1.3. Adenosine as a solution for Pharmaco-Resistant Epilepsy

Exploring endogenous inhibitory mechanisms is a nature-based approach to try to answer pharmaco-resistant epilepsy. One endogenous anticonvulsivant mechanism is the seizure-induced release of the neuromodulator adenosine.

Before going deeper into adenosine anticonvulsivant properties, it is important to integrate adenosine in its biological and biochemistry roles. Adenosine is a purine ribonucleoside (Figure 5.), one of the building blocks of life from an evolutionary and structural point of view. It was likely part of the “primordial soup” at the origin of life on Earth (Oro, 1961). Therefore it is not surprising that adenosine is an integral component of compounds essential for basic biochemistry and mitochondrial bioenergetics, such as energy transfer (ATP and ADP), nucleic acid structure (DNA and RNA (including poly-A tails), intracellular signaling (cAMP) and others. Therefore, it is an ubiquitous molecule present in all cells. Later on, in evolution, adenosine roles spanned over a wider range of functions (Dunwiddie and Masino, 2001), including the cardiovascular system, the immune system, the gastrointestinal tract and the nervous system, which is of particular interest in the context of pharmaco-resistant epilepsy.

Adenosine role as an endogenous modulator of neuronal excitability took more than 50 years to be established (Dunwiddie and Hoffer, 1980; Fredholm and Hedqvist, 1980) after the first observation from Drury and Szent- Györgyi (1929). Nowadays, its relevance in the sleep/arousal system, cognition and memory, neuronal maturation and development (Ribeiro *et al.*, 2002) is well recognized such as its neuroprotective and anticonvulsivant properties (Boison *et al.*, 2010; Fredholm *et al.*, 2005a; Ribeiro, 2005; Stone *et al.*, 2009).

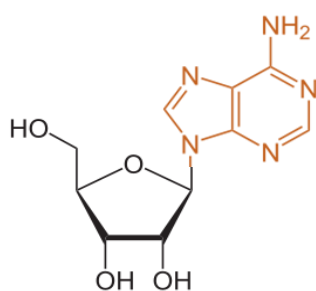


Figure 5. Adenosine chemical structure. Adenosine is composed by a purine, adenine (in orange), linked to ribose (in black) by a C1'-N9 glycosidic bond.

1.3.1. Neuromodulation by Adenosine

Being a neuromodulator, adenosine modulates the cellular properties of pre- and/or postsynaptic neurons (or glial cells) and regulates synaptic transmission and plasticity, neuronal networks and behavior (Kaczmarek and Levitan, 1987).

Its role in neuromodulation is triggered by neuronal activity and depends on its release to the extracellular space (Figure 6.). Adenosine is either directly released by neurons or glia (through adenosine equilibrative transporters) or, most frequently, originated extracellularly from released ATP, by neurons and glia, converted into adenosine by a cascade of ectoenzymes (Richardson *et al.*, 1987; Terrian *et al.*, 1989; White and MacDonald, 1990; Dunwiddie *et al.*, 1997; Zimmermann *et al.*, 2012). Extracellular ATP from astrocytes is possibly the major contributor of external adenosine concentration in physiological conditions, as confirmed in the hippocampus (Cunha *et al.*, 1996a, Koizumi *et al.*, 2003; Newman, 2003, Pascual *et al.*, 2005). Regulation of extracellular adenosine concentration is critical to healthy neuromodulation and is severely perturbed in epilepsy, which will be discussed further on.

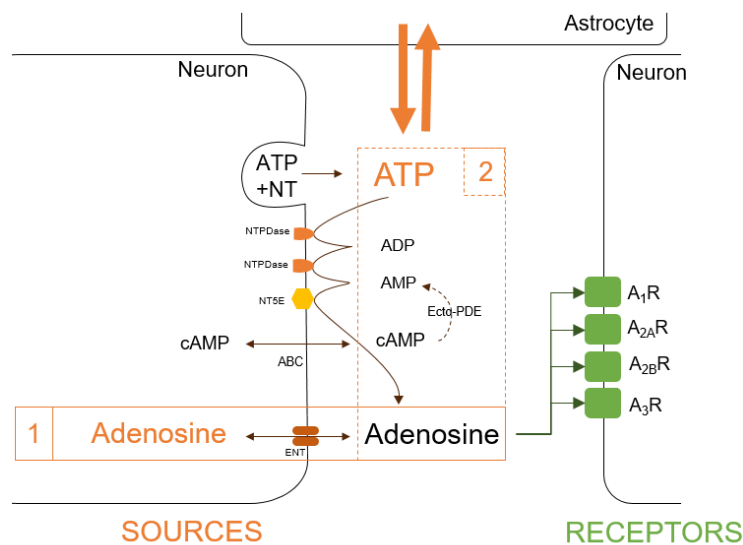


Figure 6. Adenosine sources and receptors. Extracellular adenosine can result from 1. Direct release (through adenosine equilibrative transport) or 2. Indirectly from extracellular ATP hydrolysis (by a cascade of ectoenzymes). Extracellular ATP sources may be: ATP co-release from neurotransmitter-filled vesicles (pre-synaptic neuron), ATP vesicular release from astrocytes and ATP release from channels (connexin hemichannels or pannexin channels, not depicted). Extracellular adenosine exert its neuromodulatory role through receptor-dependent mechanisms, activating adenosine receptors (ARs). Intracellular metabolism of adenosine nucleotides is not shown such as extracellular degradation. Legend: ABC: ATP-binding cassette (*ABC*) transporters; ENT: Equilibrative Nucleoside Transporters; Ecto-PDE: Ectophosphodiesterase; NT5E: ecto-5'-nucleotidase; NTPDase: ecto-nucleoside triphosphate diphosphohydrolase.

Once in the extracellular space, adenosine can influence neurotransmission by acting directly onto neurons, either pre-, post- or peri/extra-synaptically, or onto non-neuron cells by influencing glia function. It acts through receptor-dependent and independent mechanisms. Receptor-dependent mechanisms are known to modulate neurotransmitter release machinery, other ionotropic or metabotropic receptors, transporters or control of function of other neuronal modulators (Ribeiro and Sebastião, 2010). Receptor-independent mechanisms are less studied and involve transmethylation and epigenetics, mitochondrial bioenergetics and additional biochemical pathways (Boison, 2012b; Boison, 2016).

Focusing on receptor-dependent mechanisms, adenosine acts via four known guanine-binding protein (G-protein)-coupled adenosine receptors (ARs): adenosine receptor type 1, 2A, 2B and 3 (A_1R , $A_{2A}R$, $A_{2B}R$ and A_3R , respectively) (Boison and Stewart, 2009; Fredholm et al. 2001, 2011) (Figure 7.). These receptors have a distinctive pharmacological profile, tissue distribution and effector coupling, and its functioning has been extensively studied in the CNS (see Table 6.). In basal physiological conditions, the intracellular concentration of adenosine is estimated to be less than 50 nM, whereas the extracellular synaptic concentration of adenosine ranges from 25 to 250 nM (Ballarín *et al.*, 1991, Dunwiddie and Diao, 1994) with small variations in-between brain regions (Delaney and Geiger, 1996). This is sufficient to activate A_1 and A_{2A} receptors in a physiological condition, but not A_{2B} and A_3 receptors, which require higher agonist concentrations thus seen as more relevant in a pathological scenario.

As described before, these receptors can be grouped by affinity: A_1R and $A_{2A}R$ are high-affinity receptors, which have higher physiological relevance, while $A_{2B}R$ and A_3R are low-affinity receptors, gaining more relevance in a pathological scenario. ARs can also be grouped by function: activation of A_1 receptors leads to an inhibition of synaptic transmission and neuronal excitability, while activation of A_{2A} and A_{2B} receptors leads to an increase in neurotransmitter release and excitation (Sebastião and Ribeiro, 1996). The properties of A_3 receptors in the brain are still not well understood (Jacobson, 1998). It is important to note that A_3Rs display high affinity for adenosine in humans, unlike what occurs in the rat (Fredholm *et al.*, 2001), but have a low density in most tissues in humans (Ribeiro *et al.*, 2003).

In Figure 7., ARs activation is depicted along with its main signaling pathways and effects. Of special interest in the context of seizure arrest is A_1R , due to its function, density and distribution.

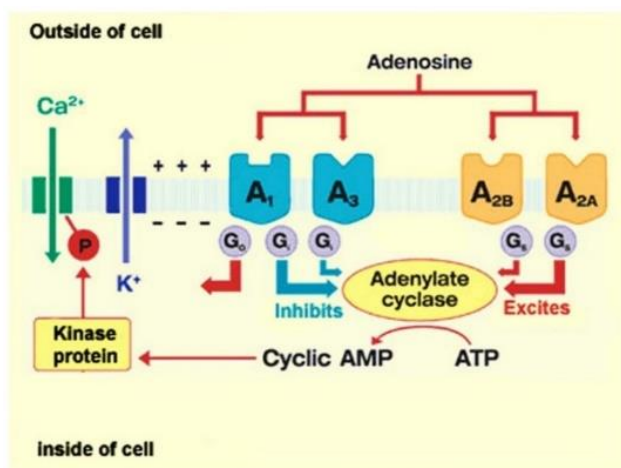


Figure 7. Adenosine receptor signaling. Adenosine receptors are G-coupled proteins negatively (A_1R , A_3R) or positively ($A_{2A}R$, $A_{2B}R$) coupled to adenylate cyclase (AD). Inhibition of AD leads to lower cAMP concentrations, which prevents protein kinase from phosphorylate Ca^{2+} channels, a step required for Ca^{2+} influx. A_1R and A_3R also directly potentiate potassium channels opening. $A_{2A}R$ and $A_{2B}R$ potentiate AD activity, leading to increased Ca^{2+}

influx, which is necessary for neurotransmitter vesicle release synchronization, augmenting the signal in the synapse, therefore being called excitatory. From Ham and Evans, 2012.

Table 6. Adenosine receptor characterization in the CNS

Receptor Type	A_1R	$A_{2A}R$	$A_{2B}R$	A_3R
Adenosine Affinity	70 nM	150 nM	5100 nM	6500 nM
G-protein coupling	G_i and G_o	G_s and G_{olf}	G_s	G_{i3} and G_q
Transduction Mechanisms	Inhibits AC Inhibits Ca^{2+} channels Activates GIRKs Activates PLC	Activates AC Activates Ca^{2+} channels Inhibits Ca^{2+} channels	Activates AC Activates PLC	Inhibits AC Activates PLC Increases intracellular Ca^{2+}
Physiological Actions	Inhibits synaptic transmission Hyperpolarizes neurons	Facilitates transmitter release Inhibits transmitter release	Increases cAMP in brain slices Modulation of Ca^{2+} channel function	Inhibits A_1R mediated responses (1) Inhibits mGluR mediated responses(2);
High Abundance	Hippocampus Neocortex Cerebellum Spinal cord	Striatum Olfactory bulb	-	-
Medium/Low Abundance	Amygdala Olfactory bulb Striatum Thalamus Substantia nigra	Hippocampus Neocortex Thalamus	Uniform low level of expression	Hippocampus Cerebellum

Legend: AC: adenylate cyclase; GIRKs: G-protein–dependent inwardly rectifying K^+ channels; mGluR: metabotropic glutamate receptor; PLC: phospholipase C. (1) (Dunwiddie et al. 1997); (2) (Macek et al. 1998); Adapted from (Dunwiddie and Masino, 2001; Boison 2005).

1.3.2. Endogenous anticonvulsivant mechanisms of Adenosine

Activation of A₁R is considered an inhibitory biological endogenous mechanism, which inhibits network excitability, limits the extension of seizures and mediates seizure arrest (Etherington and Frenguelli, 2004; Fedele *et al.*, 2006; Li *et al.*, 2007). A₁R is widely distributed in CNS with prominent abundance in the hippocampus, cerebral cortex, cerebellum and dorsal horn of spinal cord (Goodman and Synder, 1982; Mahan *et al.*, 1991; Reppert *et al.*, 1991; Ribeiro *et al.*, 2003) (Table 6). It can be found heterogeneously expressed within neurons in the pre- and postsynaptic density (Rebola *et al.*, 2003) as well as in non-neuronal cells such as astrocytes (Biber *et al.*, 1997), microglia (Gebicke-Haerter *et al.*, 1996) and oligodendrocytes (Othman *et al.*, 2003).

The coupling of A₁Rs to G_i/G_o proteins underlies several mechanisms by which adenosine can modulate synaptic transmission (Dunwiddie and Masino, 2001), both glutamatergic and GABAergic transmission.

In the case of glutamatergic transmission, presynaptic activation of A₁Rs causes decreased neurotransmitter release (Figure 8. A) (Fredholm and Dunwiddie, 1988). This is mediated by inhibition of adenylyl cyclase (van Calker *et al.*, 1979), PKA decreased activity and consequent reduction in neurotransmitter release probability by inhibiting calcium (Ca²⁺) influx through voltage-gated channels (MacDonald *et al.*, 1986; Schubert *et al.* 1986; Barrie and Nicholls, 1993; Yawo and Chuhma, 1993; Ribeiro 1995; Wu and Saggan, 1994; Cunha, 2001; Gundlfinger *et al.*, 2007), or by interfering directly with the release process (Scanziani *et al.*, 1992; Thompson *et al.*, 1993). At the same time, adenosine has been shown to function post-synaptically (Figure 8. B), which results in resting membrane potential hyperpolarization (Ponce *et al.* 1996, Ehrenguber *et al.* 1997), through activation of a series of downstream potassium (K⁺) channels, including G-protein-coupled, inward-rectifying K⁺ channels (GIRKS) (Segal, 1982; Greene and Haas, 1985; Trussell and Jackson, 1985; Gerber *et al.* 1989) and control burst-like activity in CNS (Dragunow, 1988). During seizures, A₁R-dependent activation of potassium channels increase the cell membrane conductance and has a shunting effect on GABA_AR currents, significantly attenuating the depolarizing GABA_AR responses and also reducing the extent of the after-discharge phase of the seizure (Ilie *et al.* 2012).

A₁R activation also reduces long-term changes in synaptic efficiency (de Mendonça and Ribeiro, 1997), activates phospholipase C (PLC)/Protein kinase C (PKC) pathway (Kendall and Hill, 1988) and can also modulate the excitatory effects of post-synaptic glutamate receptors (Takigawa and Alzheimer, 2002). It has been observed that A₁R activation induces AMPAR internalization and long-lasting synaptic inhibition in rat hippocampal CA3-CA1 synapses (Chen *et al.*, 2014). In addition to these synaptic effects, A₁ receptors are believed to provide beneficial extra-synaptic effects, which are based on

a decrease in brain metabolism (Haberg *et al.*, 2000) and the control of astrocyte function (see van Calcar and Biber, 2005).

These neuromodulatory A₁R actions result in a reduction of neuronal excitability and constitute an important neuroprotective role of adenosine during excitotoxic events such as hypoxia/ischemia or increased neuronal firing (de Mendonça *et al.* 2000).

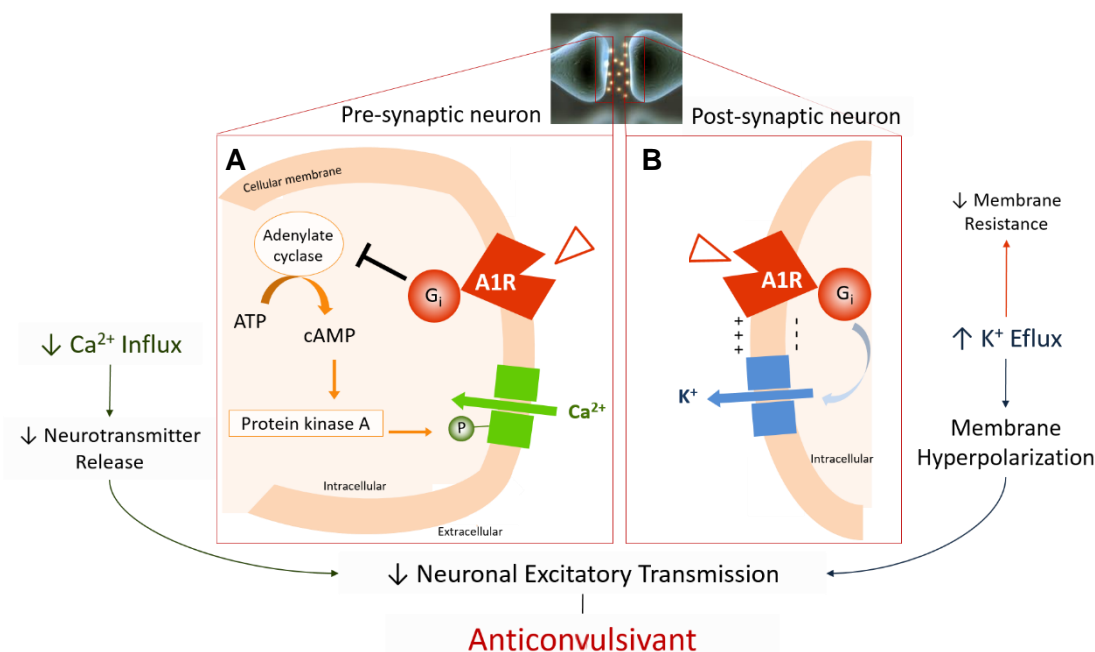


Figure 8. Adenosine anticonvulsant mechanisms through A₁R activation.

A. Pre-synaptic A₁R activation leads to decreased neurotransmitter release while **B.** Post-synaptic A₁R activation leads to membrane hyperpolarization, both contributing to decreased neuronal excitability.

In the case of GABAergic transmission, there is substantial evidence pointing towards a lack of effect of A₁R activation on hippocampal phasic GABAergic transmission (Dolphin and Archer 1983; Lambert and Teyler, 1991; Yoon and Rothman, 1991; Cunha and Ribeiro 2000a; Rombo *et al.*, 2014). Despite the absence of direct A₁R modulation on phasic GABA communication in adult hippocampus (pyramidal cells and interneurons), it has been shown that A₁R activation suppresses tonic GABA_A currents in hippocampal pyramidal cells and also in CB₁R⁺/CCK⁺ interneurons (not in CB₁R⁻/CCK⁻ interneurons) (Rombo *et al.*, 2016). Suppression of tonic GABA_A currents leads to increased synaptic transmission. In pyramidal cells, it may be a protection mechanism to prevent pyramidal cell shutdown when A₁R activation is high. In interneurons, increased synaptic transmission implies increased inhibition of pyramidal cells (Figure 9), contributing to an A₁R-mediated inhibitory role.

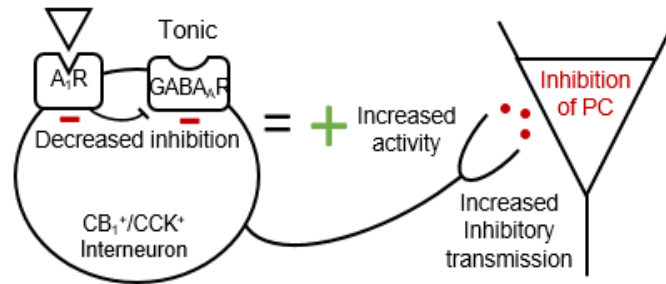


Figure 9. A₁R-mediated action upon GABAergic transmission into CA1 pyramidal cells and interneurons. Adapted from Rombo *et al.*, 2016.

Tonic but not phasic GABAergic transmission modulation by adenosine may be a protective mechanism: phasic transmission can occur “normally” but there is an increased inhibitory tonus of the system, which decreases the probability of a seizure onset, in the case of uncontrolled firing activity.

Other receptor systems, commonly associated with the control of GABA responses, are also influenced by A₁R activation, such as CB₁R-mediated control of GABA release (Sousa *et al.*, 2011) and enhancement of GABA released caused by vasoactive intestinal peptide (VIP) in hippocampal nerve terminals (Cunha-Reis *et al.* 2008).

These regulatory mechanisms of A₁R are consistent with the neuroprotective actions of adenosine in the adult brain and may confer an important developmental control of excitation during neuronal maturation.

In contrast to A₁ receptors, A_{2A} receptors are coupled to stimulatory G_s proteins - increasing adenylate cyclase activity and raising intracellular levels of cAMP - or Golf proteins (Corvol *et al.*, 2001). They have a much more restricted distribution in the brain (see Ribeiro *et al.*, 2003), being characteristic of dopamine enriched areas (e.g.: striatum) (Svenningsson *et al.*, 1997). In the hippocampus, A_{2A} receptors have been shown to increase synaptic transmission and excitability (Sebastião and Ribeiro, 1992; Cunha *et al.*, 1994a), synaptic plasticity (de Mendonça and Ribeiro, 1994) and the evoked release of acetylcholine (Cunha *et al.*, 1994b), glutamate (Lopes *et al.*, 1999) and GABA (Cunha and Ribeiro, 2000). Less is known about the contribution of the low-affinity A_{2B} and the low-density A₃ receptors. Given the low-affinity of A_{2B} receptors for adenosine, it is suggested that they have an important role in pathological conditions when extracellular adenosine concentration rises (see Fredholm, 1997).

The A₃ receptors are found mostly in peripheral tissues and have been extensively studied in cells from the immune system (e.g. Rankumar *et al.*, 1993). In the brain, its role is still controversial.

1.3.3. Adenosine (Dys)Function in Epilepsy

The endogenous inhibition of neuronal excitability by adenosine depends on the level of adenosine as well as the expression level of receptor subtypes in a given cell type or subregion of the brain. Bearing this in mind, it becomes easy to understand that neuroprotection through A₁R activation may fail if there is 1. low adenosine in the extracellular space or 2. low A₁R density. In fact, it has been observed that, in epilepsy, the adenosinergic system is dysfunctional in these two main conditions and its repair is sufficient to protect against seizures (see Table 7). Low adenosine levels in the extracellular space are largely due to a failure of astrocyte-based adenosine-cycle. Astrocytes are the major responsible for extracellular adenosine levels regulation, both in providing (through ATP) and re-uptaking adenosine, regulated by intracellular astrocytic adenosine kinase (ADK; EC 2.7.1.20) (a phosphotransferase that converts adenosine into adenosine-5'-monophosphate (AMP)). In pharmaco-resistant epilepsy, astrogliosis is one of the hallmarks and impaired adenosine levels regulation strengthens the importance of astrocytes in adenosine dysfunction in epilepsy.

Focusing in pharmaco-resistant-epilepsy, adenosine brings hope since it was shown to successfully suppress pharmaco-resistant seizures (Gouder et al. 2003). Adenosine is a special candidate for pharmaco-resistant epilepsy since it is an endogenous molecule, so it is not expectable to be a substrate for drug efflux.

Table 7. Adenosine (Dys)Function in Epilepsy

Epilepsy		
Function	Dysfunction	Repair
After the onset of a seizure, ↑ Adenosine levels remain elevated postictally to activate A ₁ R as an endogenous anticonvulsivant mechanism ⁽¹⁾ to prevent further seizure development and/or propagation	↓ A ₁ R density ⁽²⁾ ↑ A ₁ R density and mRNA ⁽³⁾	A ₁ R activation ⁽⁸⁾
	↑ A _{2A} R mRNA ⁽⁴⁾ ↓ A _{2A} R mRNA ⁽⁵⁾	A _{2A} R activation (not clear since some studies show it can suppress ⁽⁹⁾ or promote ⁽¹⁰⁾ seizures)
	ADK overexpression ⁽⁶⁾	ADK inhibition ⁽¹¹⁾
	Astrogliosis ⁽⁷⁾	↑ Adenosine levels (by AATs) ⁽¹²⁾

(1) Maitre *et al.*, 1974; Winn *et al.*, 1979; Dunwiddie, 1980; Snyder *et al.*, 1981; Dunwiddie and Worth, 1982; Burley and Ferrendelli, 1984; Haas and Greene, 1984; Haas *et al.*, 1984; Lee *et al.*, 1984; Szot and Murray, 1984; During and Spencer, 1992; Berman *et al.*, 2000; Pedata *et al.*, 2001; de Mendonça *et al.*, 2000

(2) Glass *et al.*, 1996; Ochiishi *et al.*, 1999; Ekonomou *et al.*, 2000; Rebola *et al.*, 2003a,b; Rebola *et al.*, 2005; Li *et al.*, 2007

(3) Angelatou *et al.*, 1991; Angelatou *et al.*, 1993; Pagonopoulou *et al.*, 1993; Psarropoulou *et al.*, 1994; Vanore *et al.*, 2001; Aden *et al.*, 2004; Tchekalarova *et al.*, 2005

- (4) Rebola *et al.*, 2005
- (5) Aden *et al.*, 2004
- (6) Gouder *et al.* 2004; Shen *et al.* 2011; Li *et al.*, 2008; Aronica *et al.*, 2011; Masino *et al.*, 2011
- (7) Gouder *et al.* 2004; Tian *et al.*, 2005; Binder and Steinhäuser, 2006; Boison, 2008
- (8) Barraco *et al.*, 1984; Young and Dragunow, 1994; Rauce and Rüthrich, 1995; Gouder *et al.*, 2003; Fedele *et al.*, 2006
- (9) Sarro *et al.*, 1999; Huber *et al.*, 2002
- (10) Zeraati *et al.*, 2006; Hosseinmardi *et al.*, 2007
- (11) Gouder *et al.* 2004
- (12) Boison, 2009

However, treatment of PRE with adenosine or its analogues is clinically limited due to strong systemic side effects, mainly peripheral cardiovascular (slowing of the heart beat, vasoconstriction) (Monopoli *et al.*, 1994; Gouder *et al.*, 2013). Inhibition of cardiovascular activity is so relevant that adenosine is clinically administered as an antiarrhythmic in the short pharmacological treatment of paroxysmal supraventricular tachycardia (elevated heart rate) (Riccardi *et al.*, 2008). For this reason, focal delivery of adenosine into the brain has been pursued with many techniques now developed and tested in animal models. They are called Adenosine Augmentation Therapies and can be divided into three main groups: 1. Polymer-based adenosine deliver (ethylene vinyl acetate copolymers, silk-based polymers), 2. Encapsulated cell systems (encapsulated fibroblasts, encapsulated myoblasts) and 3. Stem cells (mouse embryonic stem cells, human mesenchymal stem cells) (Boison, 2009a,b, 2012a). Despite being successful, focal therapies still imply a major economic cost (in development and application) and feasibility (surgery), when compared to adenosine systemic administration.

1.3.4. MRS5474 – a new promising adenosine derivative

If an orally administered drug could act as an A₁R agonist without its known the side effects, that would be ideal. To do so, it would be necessary to develop an adenosine derivative selective towards the central nervous system, instead of the peripheral system. Exploring a target specific mechanism that only happens in the CNS could be an answer (for example, the specific modulation of tonic GABA_AR conductance of a subset of interneurons by A₁R activation (Rombo *et al.* 2016).

With this goal, many adenosine derivatives have been developed and characterized in the past (reviewed by Jacobson *et al.*, 1992). Recently, Kenneth Jacobson's team derived adenosine analogues by N⁶-methanocarbon substitution of 4'-truncated adenosine and screened for human A₁R, A_{2A}R and A₃R selectivity, affinity and full agonism (Tosh *et al.*, 2012). The goal was to design a selective and potent human A₁R agonist to be used in various conditions treatment, including epilepsy. Upon *in vitro* testing for selectivity, affinity and full agonism, one adenosine derivative, MRS5474 (2-Chloro-N⁶-dicyclopropylmethyl-4'-truncated(N)-methanocarpa-adenosine), was selected as the most promising candidate to *in vivo* testing along with a classical highly selective A₁R agonist CCPA (2-Chloro-N⁶-cyclopentyladenosine) (K_i=0.4 nM) (Lohse *et al.* 1988; Alnouri *et al.*, 2015).

MRS5474 (Figure 10.) is described as a moderately selective A₁R-full agonist. MRS5474 affinity to hARs was assessed by radioligand binding assays: the inhibition constant (K_i) of MRS5474, relative to the binding of selective AR agonists to human AR stably expressed, is shown in Table 8. MRS5474 presented high affinity for hA₁R (K_i = 47.9 ± 10.5 nM), 10 fold-selectivity for hA₁R compared to hA₃R, and low affinity to hA_{2A}R (K_i = 3950 ± 410 nM). hA_{2B}R was not included in the initial pharmacological screen since activity within the class of (N)-methanocarpa nucleosides was previously noted to be very weak or absent at the hA_{2B}AR (Jacobson, 2000; Tosh *et al.*, 2012b). Functionally, MRS5474 was observed to be a full agonist, with hA₁R-mediated adenylyl cyclase activity comparable to CCPA (94.3±5.3%), as measured by cAMP production. Later, MRS5474 affinity to hA_{2B}R was studied through the same functional assay. MRS5474 was observed to be nearly inactive through hA_{2B}R when compared to the non-selective AR agonist NECA (10 µM) (13.7±4.4%). Thus, the A₁R selectivity of MRS5474 was maintained within the entire AR family.

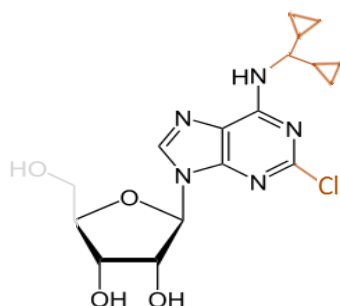


Figure 10 Adenosine derivative MRS5474 chemical structure (N⁶-dicyclopropylmethyl-4'-truncated(N)-methanocarpa-adenosine). Adenosine backbone is colored in black (gray for 4'-truncated group) and N⁶-substitution colored in orange.

Table 8. MRS5474 properties as a promising hA₁R agonist

Property	hA ₁ R	hA ₃ R	hA _{2A} R	hA _{2B} R
K _i (nM) ^a	47.9±10.5	470±15	3950±410	nd
Affinity	High	Moderately -Low	Low	Very weak or absent in within the class of (N)- methanocarpa nucleosides ^c
Selectivity	10-fold more selective to hA ₁ R than hA ₃ R		Not selective	
Full agonism ^b	94.3±5.3% Compared to CCPA			13.7±4.4 Compared to NECA

Legend:

nd – not determined

a - Determined by radioligand binding assays; hAR radioligands: hA₁R: [³H]R-PIA, hA_{2A}R: [³H]CGS21680, hA₃R: [¹²⁵I]-AB-MECA; NECA as non-specific hAR agonist, and membrane preparations from Chinese hamster ovary (CHO) cells (A₁R and A₃R) or human embryonic kidney (HEK) 293 cells (A_{2A}R) stably expressing a hAR subtype;

b – Functional data determined at a single concentration (10 µM) in an assay of adenylate cyclase (A₁R-induced inhibition of cyclic AMP production was the final measure in CHO cells stably expressing the receptor, standard full agonist used: CCPA)

c - Activity within the class of (N)-methanocarpa nucleosides was previously noted to be very weak or absent at the hA_{2B}AR (Jacobson, 2000; Tosh et al 2012b). Adapted from Tosh et al. 2012a

Moderate selectivity, high affinity and full agonism (compared to CCPA) of hA₁R by MRS5474 made it a promising candidate to test its anticonvulsant properties *in vivo* in a pharmaco-resistant model of epilepsy along with other classical epilepsy models. It is important to note that MRS5474 binding properties were studied in human ARs. On one hand, it increases translational power since the final goal is to know how MRS5474 interacts with ARs to produce an antiepileptic effect in humans. On the other hand, *in vivo* testing is not performed in humans in this phase, but rather on rodents, and adenosine receptors pharmacological properties are species-dependent (Alnouri et al. 2015).

In vivo anticonvulsant testing

MRS5474 was tested in electrical-induced (6 Hz and Maximal Electrical Shock (MES) model) and chemically-induced (subcutaneous administration of metrazol (pentylentetrazol) (scMET) model) models of epilepsy in kindled adult male (CF-1) albino mice along with a classical A₁R agonist CCPA. Toxicity of the drug was measured through the rotarod test (considered toxic if the mice fell more than 3 times per 1 minute) and named behavioral toxicity. Performance of MRSS5474 in both

aspects (anticonvulsant properties and behavioral toxicity) make it an interesting drug for treatment of pharmaco-resistant epilepsy.

MRS5474 displayed efficacy in a pharmaco-resistant model of epilepsy, 6 Hz, in mice (Table 9.): at dose 2.74 mg/kg half the animals were protected against provoked seizure. Interestingly, the toxicity observed in A₁R activation with selective agonists as CCPA was not observed with MRS5474 in the active dose range, around 3 mg/kg, nor up to the tested dose of 30 mg/kg, administered intraperitoneally. The therapeutical window for MRS5474 is thus wider than compared with CCPA. At dose of 0.12 mg/kg of CCPA, half the animals were protected, but behavioral toxicity was seen at 0.84 mg/kg. Not only the relation of toxicity dose/effector dose is higher for MRS5474 (>10 in MRS5474, 7 in CCPA) but the dose administration admitted error is bigger in MRS5474 since we are dealing with higher doses. At a similar dose that provided protection against a provoked seizure for half the animals in 6 Hz model, both CCPA and MRS5474 were not able to protect the animals against a provoked seizure in both MES model and scMET model.

Table 9. Anticonvulsant activity in mice of A₁R agonists

Compound ^a	Behavioral toxicity TD ₅₀ (mg/kg)	6 Hz model ED ₅₀ (mg/kg)	MES model ^b (dose)	scMET ^b model (dose)
MRS5474	>30 ^d	2.74 ^c	1 out of 4 (3 mg/kg)	No protection (3 mg/kg)
CCPA	0.84 ^c	0.12 ^c	1 out of 4 (1 mg/kg)	1 out of 4 (1 mg/kg)

^aadministered i.p.

^bqualitative results, expressed as number of animals protected from convulsions

^cmeasured at 1 h (time of peak of effect) post injection, dose range for MRS5474 was 0.75 – 10 mg/kg

^dNo rotarod toxicity at 30 mg/kg. Toxicity index (TD₅₀) was defined as the dose at which half the animals would fall more than 3 times per 1 minute in the rotarod test.

The 6 Hz and MES model are electrically-induced epilepsy models, where the mice are kindling (shocked with electrical shocks in the cornea for 1 week until development of epileptic status, under ethical conditions). The authors did not consider absence (or near absence) of anticonvulsant effect in MES model and scMET model (traditional epilepsy models) a discouraging property of MRS5474. On the contrary, they agree the unique response profile of MRS5474 (inactive in MES and scMET, active in 6 Hz and corneal kindling models) and its A₁R binding properties make it a potential candidate to treat drug-resistant epilepsy. Traditional anti-epileptic drugs (AEDs) carbamazepine, lamotrigine, phenytoin and topiramate are Na⁺ channel blockers, which have strong efficacy in the MES model (White *et al.*, 2002). They are either inactive or only partially

efficacious in the 6 Hz model. On the other hand, newer AEDs having different mechanisms of action, such as levetiracetam and retigabine, are potent and efficacious in the 6Hz model, which makes the 6 Hz model a model for identifying compounds that potentially target drug-resistant epilepsy (Barton *et al.*, 2001). The kindling models are useful in searching for drugs to treat complex partial seizures, because kindled seizures not only provide an experimental model of focal seizures but also a means of testing drugs to stop seizure spread and generalization from a focus (Lothman *et al.*, 1988).

Even though the analysis for behavioral toxicity is a very broad one (only analyses the motor activity on the rotarod), the fact that CCPA has impaired the overall physical performance of the mice and MRS5474 did not poses a very interesting case to figure out why MRS5474 presents an *in vivo* antiepileptic role without the A₁R activation typical side effects.

The unique activities of MRS5474 in the 6Hz and corneal kindled mouse model and its overcoming the limitations of other A₁R agonists (i.e. clear separation of anticonvulsant activity and toxicity) make it an attractive AED candidate for additional testing.

1.4. The Hippocampus: a model for antiepileptic drug testing

To establish the synaptic target of a putative therapeutic drug, as MRS5474, a suited model needs to be chosen. The hippocampus is a very interesting brain structure, not only for this specific work, but also for many others since “[it] has something for everyone” (Andersen *et al.*, 2007).

Indeed, the hippocampus provides a unique framework to study processes of varying level, ranging from the biochemical characterization of cell biological machinery, neurotransmitter receptors and ion channels to processes as synaptic transmission, neuronal plasticity, neurogenesis and higher level functions that result from micro- and macro-circuitry as memory and behavior. The basis of neurological conditions such as epilepsy or Alzheimer’s disease can also be studied in the hippocampus, since it is a brain area where pathological processes, as hippocampal sclerosis, occur. Its cellular arrangement in defined layers captured the attention of early scientists and facilitates structural analyses since different neuronal cells and connections are easily pinpointed, which is not the case for other brain regions as the cortex. Moreover, studying the hippocampus has provided general concepts of neural functioning (e.g., the neuron doctrine) that also apply in other areas of the central nervous system, making it a robust model for Neuroscience research.

For these reasons, the pyramidal neurons of the hippocampus have become the most intensively studied neurons in the brain. In this project, our focus lies in understanding how MRS5474 is able to change excitatory synaptic transmission in the excitatory neurons at the hippocampus. This is a first step to then later evaluate how MRS5474 changes excitatory and inhibitory synaptic transmission of inhibitory neurons (interneurons) in a physiological and pathophysiological state. To better understand the model used herein, hippocampal structure and circuitry will be briefly described, setting the ground for CA1 pyramidal neurons and interneurons electrophysiological properties to be focused upon alongside with synaptic and action potentials. It is necessary, however, to highlight the limitations of the used model in this work: acute hippocampal slices, when comparing and inferring from the obtained results.

1.4.1. Hippocampal anatomy and circuitry

From the beginning of brain research, the hippocampus has played a central role. Its elegant and curved structure, visible even to the ancient anatomists, captured their attention. The term hippocampus (Latin for seahorse, derived from the Greek word hippos meaning “horse” and kampos meaning “sea monster”) was coined by the anatomist Giulio Cesare Aranzi, in 1587, because of its similarity to the tropical fish seahorse (Figure 11. Center). Not only this structure resembles a seahorse, but already

around 300 BC - 300 AD, members of the Alexandrian School of Medicine noted its similarity to the coiled horns of a ram (Figure 11. Upper left) and named it Cornu Ammonis, which means Ammon's horn, the horn of a ram shaped Egyptian god (Andersen *et al.*, 2007). However, this term endured in the literature as describing the hippocampus circuitry areas Cornu Ammonis (CA) 1, 2 and 3, since they too resemble the horns of a ram (Figure 11. Right).

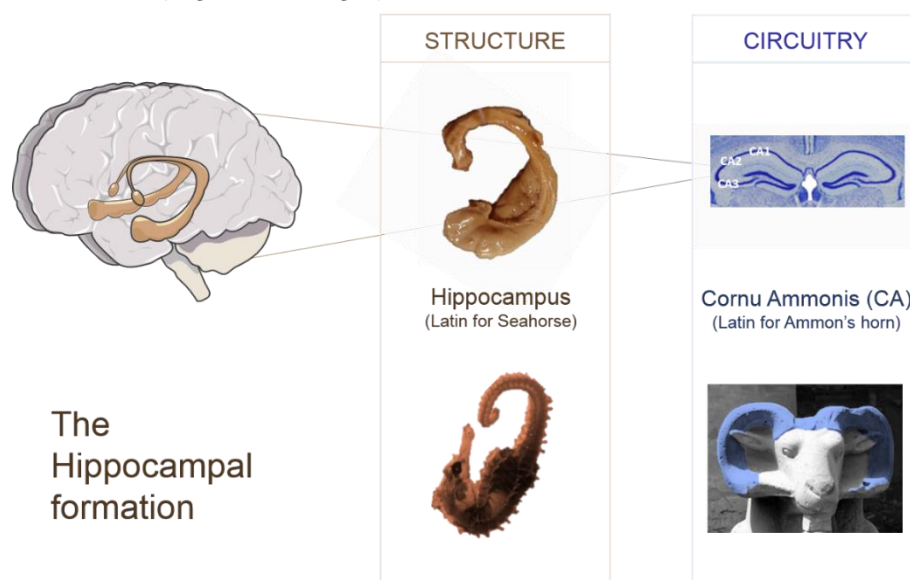


Figure 11. Origin of hippocampus nomenclature from shape similarity.

Left: Representation of the hippocampus formation in the human brain (adapted from **Servier Medical Art**, Powerpoint Image Bank). **Center:** Structure of human hippocampus dissected free (top) compared to a specimen of *Hippocampus leiria*, common name seahorse (bottom) (preparation by László Seress in 1980). **Right:** Mouse hippocampus circuitry organization (evidenced by Nissl-stained coronal slices of mouse brain) (adapted from DeFelipe, 2011) (top) compared with the horns of the ram shaped ancient Egypt god Ammon (bottom). (Adapted photography from the Karnak Temple, Luxor, Egypt). Images are not in scale.

Defining which cytoarchitectonically distinct brain regions comprise the hippocampus is a matter of debate. Consensually, the hippocampal formation is seen as group of distinct but related brain regions that act together as one functional system. These regions include the entorhinal cortex (EC), dentate gyrus (DG), hippocampus proper, subiculum, presubiculum and parasubiculum. These regions are linked, one to the next, by a large unidirectional neuronal pathway (Amaral and Witter, 1989). Often, as in this thesis, the word hippocampus is used to refer to the structure comprising the hippocampus proper and DG. The hippocampus proper can be further divided in the Cornu Ammonis areas: CA1, CA2 and CA3 area (Lorente de Nó, 1934). Functionally, the hippocampus is a microcircuit, designated trisynaptic circuit (Andersen *et al.* 1971), with three main connections: EC → DG; DG → CA3; CA3 → CA1 (see Figure 12). The trisynaptic circuit is a unidirectional excitatory feedforward glutamatergic circuit. CA1

pyramidal cells provide the final output of this circuit and are highly regulated by the activity of inhibitory neurons, interneurons, before the final output is send.

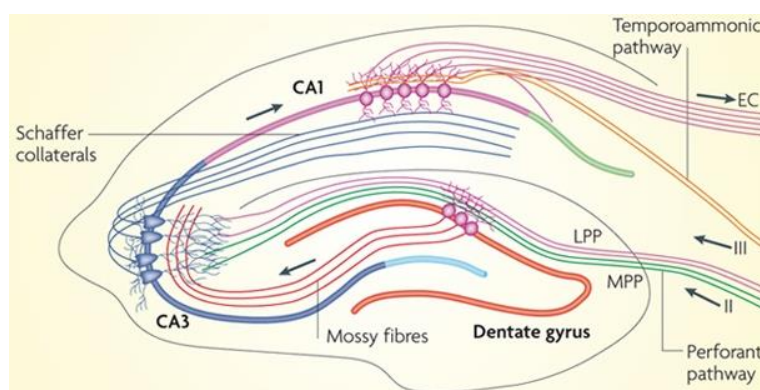


Figure 12. An illustration of the rodent hippocampal circuitry. The traditional excitatory trisynaptic pathway (entorhinal cortex (EC)–dentate gyrus–CA3–CA1–EC) is depicted by solid arrows. The axons of layer II neurons in the entorhinal cortex (EC) project to the dentate gyrus (DG) (red) through the perforant pathway (PP), including the lateral perforant pathway (LPP) (purple fibers) and medial perforant pathway (MPP) (green fibers). The dentate gyrus sends projections to the pyramidal cells in CA3 through mossy fibers (red). In CA3 are (blue), pyramidal cells also receive direct projections from EC layer II neurons through the Perforant Path. CA3 pyramidal neurons relay the information to CA1 pyramidal neurons through Schaffer collaterals (blue). CA1 receives also receives direct input from EC layer III neurons through the Temporoammonic pathway (TA) (orange). CA1 pyramidal cells provide the final output of this circuit to back to the EC. The dentate granule cells also project to the mossy cells in the hilus and hilar interneurons, which send excitatory and inhibitory projections, respectively, back to the granule cells. Adapted from Deng *et al.*, 2010.

The function of hippocampus was highly debated in the 18th and 19th centuries, but is nowadays well established to be involved in memory acquisition, spatial learning and navigation is (Stark, 2007). In physiological conditions, the recurrent connections in CA3 area work as an auto-associative network and have been proposed as essential for reconstructing already encoded patterns and retrieving previous experiences (Hasselmo *et al.* 1995; Nakazawa *et al.* 2002; Rolls, 2007), which is fundamental for its role in memory. It has also been observed that the CA3 region generates sharp waves and high-frequency oscillation EEG patterns implicated in memory consolidation (Buzsáki and Silva, 2012). However, the same properties that allow CA3 pyramidal cells to generate high-frequency oscillations implicated in memory consolidation, also make them prone to generate ictal-activity. These are two: intrinsic bursting properties and recurrent excitatory synaptic connections.

CA3 pyramidal cells have an intrinsic burst propensity, apparently based on a relatively high density of calcium channels in their proximal dendrites (Engel *et al.*, 2007). Membrane depolarization not only may trigger conventional sodium action potentials, but also may open these calcium channels. The calcium influx causes a more prolonged depolarization of the cell, driving additional action potentials in a “burst”. When these bursts occur, they provide a potent drive not only to CA1 targets, but also to neighboring CA3 pyramidal cells. This occurs due to the recurrent excitatory synaptic connections CA3 pyramidal cells establish with one another. A gradual recruitment of CA3 neuronal activity can thus lead to synchronized burst discharges. These can have a physiological role in establishing rhythms but a minor imbalance in inhibition of CA3 neurons can easily result in ictal-activity that is relayed to CA1 pyramidal cells.

However, CA3 area shows reluctance to generate seizures and continues to generate only brief interictal activity during intense CA1 seizures (Schwartzkroin, 1993). This happens due to two main reasons: strong inhibitory circuitry in CA3 area and burst-generation mechanism also antagonizes its genesis. The generated burst is thought to occur due to calcium channels, as previously stated. However, these cells also present a very effective mechanism for turning off these bursts – the after-hyperpolarization generated by calcium-dependent potassium channels (as explained in section 1.2.). Thus, the very mechanism of burst generation – calcium influx – also involves a self-limiting process (the calcium activated hyperpolarization). Simultaneously, the same mossy fiber input that activates CA3 cells also drives local interneurons very effectively so that CA3 cells are tonically inhibited by a variety of interneurons subtypes.

1.4.2. CA1 pyramidal cells

CA1 pyramidal cells, on the contrary, have a high susceptibility to generate seizure activity. But only in the presence of a synchronous synaptic ‘drive’ from CA3 neurons (Barbarosie and Avoli, 1997).

Similarities between CA3 and CA1 pyramidal cells include its morphology, a triangular soma shape, by which they are named after, and other key structural features, as a single axon, a large apical dendrite, multiple basal dendrites and the presence of dendritic spines (Spruston, 2008).

Differences between CA3 and CA1 pyramidal cells are more extensive. They include less population heterogeneity, non-bursting activity, little or absent recurrent excitatory connections and a lower level of inhibition by interneurons. These characteristics, non-bursting activity and little or absent recurrent excitatory connections, might lead us to think that CA1 pyramidal cells would be less prone to experience spontaneous seizure activity. In fact, that holds true to ictal-activity generation, but once that information is received from CA3 neurons, CA1 pyramidal cells do not possess the same level of

protective mechanisms that prevent seizure activity in CA3 neurons (strong inhibitory activity and accentuated after-hyperpolarization current).

Along with subiculum neurons, CA1 pyramidal cells constitute the output cells of the trisynaptic circuit. If seizure activity is generated in CA1 susceptible neurons, it will then be relayed to other structures, as the EC, initiating a seizure foci. Thus, the functional integrity of hippocampal output neurons represents a critical control point in temporal lobe epileptogenesis (Barbarosie and Avoli, 1997).

For this reason, studying how antiepileptic drugs affect CA1 pyramidal neurons activity has become a common method to understand its mechanisms of action. Nowadays, we take advantage of the fact that CA1 pyramidal cells have been extensively studied since the 19th century and its electrophysiological properties are well characterized (Henze and Buzsáki, 2001). Their firing pattern is commonly regular spiking, showcasing accommodation: frequency of action potentials fired is lower when the number of action potentials is increased. Typically, the resting membrane potential is around -70 mV, the firing threshold around -55 mV, AP amplitude around 100 mV and lasts around 1-2 ms, with interspike interval varying with number of action potentials fired.

1.4.3. Interneurons

The major basis for local circuit communication within the CA1 area lies in interneuronal circuitry. As explained in section 1.2., interneurons present a high heterogenous population of cells, in terms of anatomy, molecular and firing pattern. They are identified by releasing GABA at their synapses. However, that information is no accessible during the experiment, so location, size and firing pattern are usually used for identification. In CA1 molecular layer, interneurons have a low distribution (only 10% are interneurons, 90% are CA1 pyramidal cells). Usually they are smaller than CA1 pyramidal cells and are characterized by high frequency activity (in the range of 80 Hz, while CA1 pyramidal cells reach 30 Hz, under physiological conditions). In CA1 region, interneurons perform a variety of operations (feed-back inhibition, feed-forward inhibition and disinhibition, see Figure 13) that are specific to determined interneuron populations.

In conjunction with CA1 pyramidal neurons, these cell not only represent the functioning of excitatory and inhibitory synaptic transmission but also have their function altered in epilepsy. For these, both CA1 pyramidal neurons and interneurons are of main interest to understand the role of a putative antiepileptic drug.

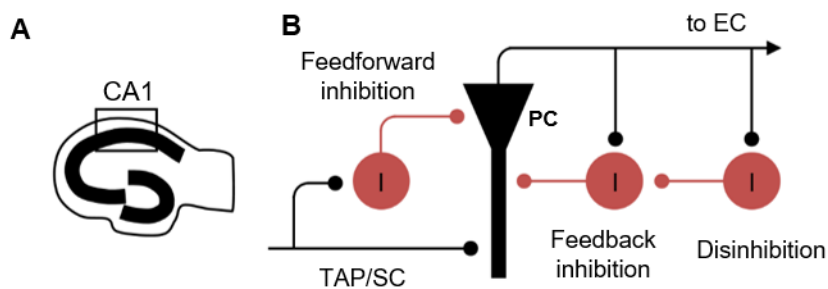


Figure 13. Hippocampal operations performed by distinct populations of CA1 interneurons (A) Schematic representation of a coronal slice of the hippocampus highlighting the CA1 region. (B) Simplistic representation of forms of feedback and feedforward operations performed by interneurons. TAP – Temporoammonic pathway; SC – Schaffer Collateral fibers; EC – Entorhinal Cortex. Adapted from Rombo, 2015.

Acute hippocampal slices

In this work, the hippocampus is a structure of great value to be studied due to the following reasons, some already mentioned:

- Is implicated in epilepsy: hippocampal sclerosis (HS) is an hallmark of TLE;
- It is well characterized - CA1 pyramidal neurons are the most studied neurons of the brain, which provide a rich reference background in which to integrate our results;
- It has well-defined cytoarchitecture shared by rats and humans – the least heterogeneous neuronal population, the somata and dendrites of pyramidal cells are arranged into well-defined layers and the extrinsic and intrinsic inputs are segregated;
- Its study has revealed general properties, usually applicable in other areas of the central nervous system;
- Acute hippocampal slices are stable for long periods; maintain their structural integrity, unlike cultures or cell homogenates; allow the study of specific circuits and brain networks in isolation; the electrophysiological recordings are more stable than *in vivo* recording since heartbeat and respiration of the experimental animal are eliminated, allowing for long periods of cellular recording; direct visualization of the slice enables the localization, identification and easy access the cells studied and also allows for drug application, which is otherwise blocked by the blood brain barrier.

The main disadvantage of using hippocampal slices is the necessary lack of neuronal activity connecting other brain structures that are no longer connected to the hippocampus. However, even though the external connections of this microcircuit are impaired, the internal connectivity remains intact.

2. AIMS

A new adenosine derivative, MRS5474, was previously shown to be a functional agonist of A_1R in overexpressing cells and presented antiepileptic properties *in vivo* in a model of Pharmaco-Resistant Epilepsy (6 Hz), without observable side-effects (being these the main obstacle to use adenosine as a pharmacological approach to treat Pharmaco-Resistant Epilepsy).

The main goal of the project is to understand the mechanism of action and the neuronal target of MRS5474. In this work, the following hypothesis was tested:

MRS5474 decreases excitatory synaptic transmission in CA1 pyramidal cells of the hippocampus through A_1R activation.

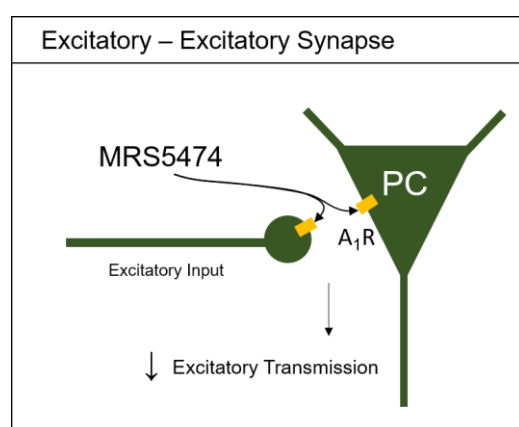


Figure 14. Schematic representation of the proposed hypothesis tested.

Decreased excitatory transmission is a strategy for seizure suppression and it is the main function of adenosine as an endogenous anticonvulsivant. However, the lack of the typical A_1R activation in the periphery (mainly in the heart), inferred from a behavioral toxicity test, argues for a more complex mechanism of action of MRS5474. If its mechanism of action provide a selective characteristic for the central nervous system, it may represent a new promising pharmacological platform for Pharmaco-Resistant Epilepsy therapeutics.

TECHNIQUES & METHODS

3. TECHNIQUES

3.1. Electrophysiological recordings in acute hippocampal slices

3.1.1. Acute Hippocampal Slice Preparation

Brain tissue was shown to be kept alive and healthy outside the body in *ex vivo* mammalian brain slices, since their synaptic activity and excitability remained similar to that observed *in vivo* (McIlwain *et al.*, 1951; Collingridge, 1967; Yamamoto and McIlwain, 1996). Following the pioneering work from McIlwain and colleagues, the brain slice preparation has become one of the most commonly used experimental preparations in Neuroscience, in particular in hippocampal research. Indeed it has been the biological support for much of our understanding of neuronal function at the cellular and synaptic level, since it presents several technical advantages for the investigation of the central nervous system (see Table 10).

Table 10. Advantages and disadvantages of hippocampal slices in Neuroscience research

Advantages
<ul style="list-style-type: none">• Shared anatomy and circuitry of hippocampus between rodents and humans• Viability Stable for long periods (6-7 hours)• Structural integrity Maintains cytoarchitecture and synaptic circuits, unlike cultures or cell homogenates• Specific circuit analysis in isolation• Stability of electrophysiological recordings More stable than <i>in vivo</i> recording since heartbeat and respiration of the experimental animal are eliminated, allowing for long periods of cellular recording• Direct visualization Enables localization, identification and easy access the cells studied, accessible for optical imaging or electrophysiological studies• Accurate control of the environment of the slice Drugs can be applied in known concentrations either to the entire slice or to selected regions, which is otherwise blocked by the blood brain barrier; drugs could also be removed from the tissue when desired
Disadvantages
<ul style="list-style-type: none">• Isolated structure Absence of afferent input from other brain structures that are no longer connected to the hippocampus• Non-optimal maintenance of tissue Compared to <i>in vivo</i>, oxygenation and resources have limited availability• Acute insult to the tissue due to slicing

Obtainment of acute hippocampal slices requires 1. Hippocampus dissection and slicing and 2. Slice recovery.

In order to preserve as much as possible the integral properties of the slice, 1. Hippocampus dissection and slicing should be performed in:

- Reduced time – prolonged delays result in deterioration of the tissue and reduced quality of the slices.
- Reduced temperature – decreases cell metabolism, delays tissue deterioration and helps maintain the consistency of the cerebral tissue;
- Reduced excitotoxic environment – higher glucose concentrations (10 mM) than reported *in vivo* (0.47 – 4.4 mM) are neuroprotective (Schurr et al., 1989; Schurr 1999; Cater et al., 2003).

Once the slices have been obtained, it is necessary to provide an adequate environment in which they can recover from the stress induced by hippocampus manipulation and slicing. To do so, 2. Slice recovery should be performed in an activity inducer environment, including:

- Increased temperature (35 °C) – promote cell metabolism functioning.

3.1.2. Patch-clamp

The development of Patch-clamp technique in the late 1970s and early 1980 has revolutionized neuroscience research. Its importance has render their inventors, Neher and Sakmann, the Nobel Prize in Physiology or Medicine in 1991 and its discovery made it possible to record the currents of single ion channel molecules for the first time, which improved our understanding of the involvement of channels in fundamental cell processes such as action potentials and neuronal activity.

Until today, electrophysiology is one of the sounding pillars of neuroscience and patch-clamp one of the most common used techniques. Described in a simple way, the patch-clamp technique allows for insight of the electrical activity of the neuron (whether a patch of the membrane or single ion channels). It is achieved by placing an electrode in the membrane patch with such close proximity that they form a single electrical unit, where we can maintain (through clamping) the membrane potential to a desired value (Voltage hold, V_h , typically -70 mV), in voltage-clamp mode. To do so, it is necessary to constantly measure the actual membrane potential and then apply the necessary electrical current to counter act it and maintain it at a fixed V_h . The necessary electrical current applied is monitored in a screen in live time and it is kept constant if the membrane potential of the cell is kept constant. Ideally, no current should be injected, which would mean that the neuron is already at the desired membrane potential. Whenever there is electrical activity in the neuron, its membrane potential will change and, consequently, the injected current will also change. In practice, we are indirectly measuring changes in voltage of the

membrane through injected current. This technique is based on Ohm's law, that states that voltage (V) is equal to current (I) times resistance (R), equation 1.

$$(eq. 1) \quad V = I \times R$$

Considering the resistance of the system to be maintained throughout the experiment, then changes in voltage are reflected by changes in current, and vice-versa. If the vice-versa situation is considered, then we are in the presence of current-clamp mode.

The key point that allowed this technique to work, apart from the electronic development, was the establishment of a tight proximity between the electrode and the membrane patch, such that they can work as a single electrical unit. That was made possible when Neher and colleagues (Neher, 1981) found out that very high resistance seals (in the order of 10-100 GΩ) can be formed between the cell membrane and the tip of a clean pipette when gentle suction is applied to the pipette interior. Along with the gigaohm seal technique, it was possible to gently pull the membrane patch with the attached pipette off the cell and study its trapped ion channels. Subsequently, it was found that the patch of membrane under the pipette tip could be removed, and once this had happened the electrode achieves direct electrical contact with the cell interior. As a result, the voltage across the entire cell membrane could be clamped, instead of the voltage across the tiny patch. This is how the technique came to be known as the whole-cell voltage-clamp.

The voltage-clamp technique is often used to study one type of channels among the many present in a given cell. When this is the case, some procedures must be done to separate the currents through the channel of interest from the currents through any other channels. This can be achieved by selectively choosing the ionic composition of the pipette and/or the bath solution; by controlling the holding potential of the cell membrane; or by using different pharmacological agents that selectively block or activate specific channels or channel groups (see Sontheimer and Olsen, 2007 for review).

Patch-clamp recordings can be performed in many different configurations that can involve the entire cell (whole-cell) or cell membrane excision and that may preserve (outside-out) or not (inside-out) the original exposure of the cell membrane to the extracellular medium. Recordings that maintain the cell structure can be performed in the cell-attached, whole-cell or in its variant, the perforated patch mode. Whereas the cell-attached mode allows only for activity of the channels inside the pipette limit to be studied, the whole-cell and perforated patch modes allow the measurements of the currents flowing through the entire population of channels in a cell membrane (see Lien et al., 1995 for review).

Typically, whole-cell mode is the most widely configuration used mode as it is easy to obtain and offers the possibility of applying modulators or drugs to the intracellular solutions in order to study signaling cascades associated to known phenomena.

4. METHODS

All experiments were conducted in accordance with the Portuguese law on Animal Care and European Union guidelines (Directive 2010/63/EU).

4.1. Animals

Male young-adult wild-type Wistar rats (3-8 weeks old, Crl:WI, strain 003) were used in all experiments. The animals were housed in the local Animal House (Animal Biosafety Level 2) with standardized humidity, temperature and a 12 hours light/dark cycle in groups of 5-8 animals and were provided conventional food and water *ad libitum*. To be anesthetized, animals were transported from the Animal House in the same type of cages (covered with the same type of bedding as before) and had access only to water before being anesthetized. Care was taken so as to reduce animal suffering and the number of used animals to the absolutely necessary.

4.2. Electrophysiology in acute hippocampal slices

Measuring electrophysiological properties of hippocampal cells or circuits is a method that can be divided into three main steps (see Figure 15):

1. Acute Hippocampal slice preparation (obtainment and recovery)
2. Electrophysiological recordings (Patch-clamp whole cell) in hippocampal slice
3. Data analysis

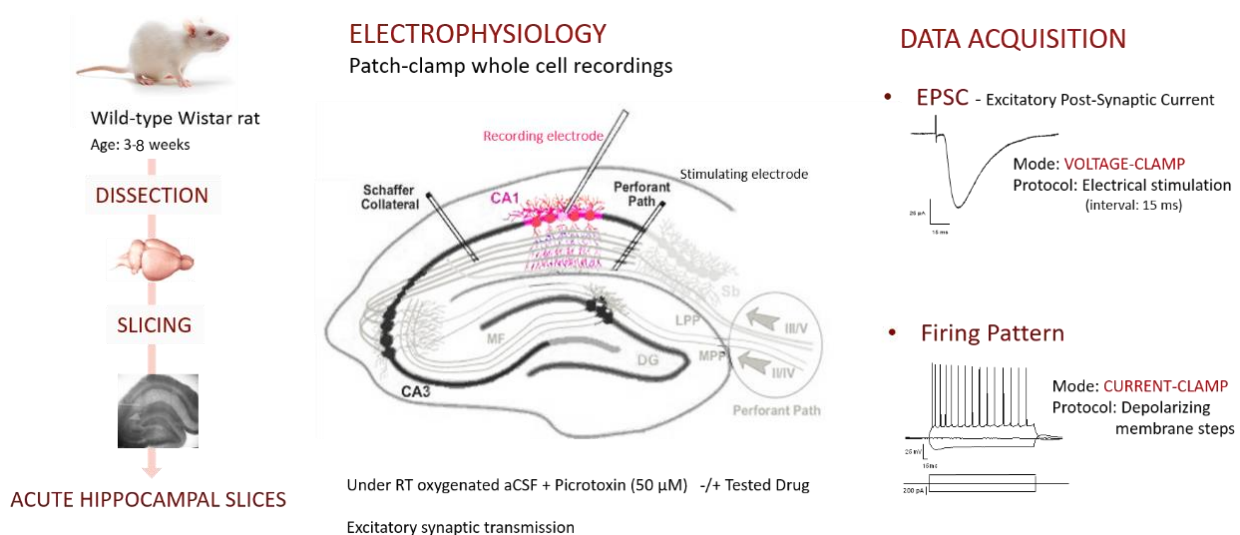


Figure 15. Diagram of methodological approach – Electrophysiology in CA1 Pyramidal Cells in rat hippocampus. Wild-type male wistar rats (aged between 3-8 weeks) were used to obtainment of the brain (through euthanasia and decapitation). The hippocampus is dissected and cut into transversal 300 μm thick hippocampal slices, under appropriate conditions. After hippocampal slice recovery from slicing, electrophysiology recordings are performed under the method of patch-clamp whole-cell configuration. The recording electrode is placed in CA1 pyramidal cells, identified with a pink color. To study excitatory synaptic transmission, these cells are electrically stimulated either through the Schaffer Collateral fiber or the performant pathway with a

stimulating electrode. Pharmacological isolation of the excitatory component of the synaptic transmission is achieved through perfusion of picrotoxin (50 μ M), a GABA_A ionotropic receptor blocker. Tested drugs are perfused into the system after stability of the electrophysiological recordings (CCPA 30 nM and MRS5474 120 nM). These can be divided into two groups: EPSPs recordings (inset) were obtained during experiments herein described and firing patterns, recorded to measure intrinsic excitability properties.

The basic principle is to obtain a transversal hippocampal slice from the adult rodent brain *ex vivo* that remains viable for several hours⁽¹⁾, typically used to study the electrophysiological properties of individual neurons or circuits⁽²⁾.

1. Preparation of hippocampal slices for electrophysiological recordings

Acute hippocampal cultures were prepared according to Rombo, 2015. Briefly described, the animals were anaesthetized^(a) (with Isoflurane, in 1,2-Propylenglycol 50% (v/v)) in an anesthesia chamber), sacrificed (by decapitation in an appropriate sized guillotine) and the brain quickly removed^(b). The isolated brain was hemisected and the hippocampi dissected^(c) and cut to obtain transverse^(d) slices (300 μ m thickness) on a Vibratome (Leica VT 1000S; Leica Microsystems, Germany), under an ice-cold^(e) (4 °C) oxygenated dissection solution containing (in mM): sucrose 110, KCl 2.5, CaCl₂ 0.5, MgCl₂ 7, NaHCO₃ 25, NaH₂PO₄ 1.25, glucose 7, pH 7.4, bubbled with 95% O₂/5% CO₂. Once the hippocampal slices are obtained, they were first incubated for 20 minutes at 35°C^(f) in oxygenated artificial Cerebrospinal Fluid (aCSF) that contained (in mM): NaCl 124, KCl 3, NaH₂PO₄ 1.25, NaHCO₃ 26, MgSO₄ 1, CaCl₂ 2, glucose 10, pH 7.4 (gassed with 95% O₂/5% CO₂), (Fredholm *et al.*, 1984). After this period, slices are allowed to recover at room temperature (RT) (22-24°C) for at least 1 hour^(g) before use in Patch-Clamp recordings.

Notes:

1- Quality of hippocampal slices is primordial to perform electrophysiological experiments

- (a) The first indications of anesthesia include the lack of a righting reflex and reduction in respiratory rate. A noxious stimulus (i.e. toe pinch) was also applied to ensure deep plane of anesthesia. If no response was noted, the animal was sacrificed by decapitation in a guillotine. Death by asphyxiation in the anesthesia chamber instead of anesthesia will compromise the quality of the hippocampal slices and it is not ethically acceptable.
- (b) It is very important to be as fast as possible in preparing the hippocampal slices. Prolonged delays result in deterioration of the tissue and reduced quality of the slices. A reasonable goal is to have cut slices separated in less than 5–10 min after the animal has been euthanized.

- (c) Gentle and minimal manipulation of tissue is crucial to avoid damaging.
- (d) The transversal cut of hippocampal slices ensures that the entire trisynaptic circuit is obtained. If the slice is cut with a different angle, the circuit might not be complete and do not establish the correct connections between regions.
- (e) The low temperature of the cutting solution decreases the cell metabolism and helps maintain the consistency of the cerebral tissue. Keeping all dissection solutions cold and performing the entire procedure over ice helps delaying the deterioration of the tissue during dissection.
- (f) After hippocampal dissection and slice preparation, it is necessary to ensure the energetic and functional recovery of the tissue.
- (g) To maximum hippocampal slice quality, it is advisable to wait 3 hours before starting the electrophysiological experiment (Fiala *et al.*, 2003).

2. Electrophysiological recordings (Patch-clamp whole cell) in hippocampal slice

Four main requirements are necessary to perform electrophysiological recordings in a setup: (1) environment: to keep the preparation healthy and administer drugs; (2) optics: to visualize the preparation; (3) mechanics: to stably position the microelectrode; and (4) electronics: to record and amplify the signal.

1. Environment

Individual dorsal hippocampal slices were placed in a submerged recording chamber (Luigs & Neumann, Ratingen, Germany) and continuously superfused by a gravitational superfusion system at 3-4mL/min with oxygenated aCSF at RT. Unless otherwise stated, drugs were added to this superfusion solution and reached the recording chamber within 2-3 minutes.

2. Optics

CA1 pyramidal cells were visually identified using a Carl Zeiss Axioskop 2FS upright microscope (Jena, Germany) equipped with a 40x immersion objective with 2 and 4 zoom (i.e. up to 160x magnification) and a differential interference contrast-infrared (DIC-IR) CCD video camera (VX44, Till Photonics, Gräfelfing, Germany).

3. Mechanics

Physical stability is obtained through a vibration isolation table in the electrophysiology rig (Luigs & Neumann, Ratingen, Germany), controlled micromanipulators (Technical Manufacturing Corporation, Germany) and fixing the individual hippocampal slices with a harp slice grid with nylon strings (HSG-5BD, ALA Scientific Instruments, Farmingdale, NY, USA) to minimize agitation.

4. Electronics

Whole-cell recordings were obtained from pyramidal cells located at CA1 stratum pyramidale of rat hippocampus.

Recording Patch pipettes were made from borosilicate glass capillaries (1.5 mm outer diameter, 0.86 mm inner diameter, GC150F-10, Harvard Apparatus, Holliston, MA, USA) in two stages on a pipette puller (PC-10 Puller, Narishige Group, London, UK). The resistances of the recording pipettes were 4-9M Ω ^(a) when filled with an internal solution^(b) containing (in mM): K-gluconate 145, HEPES 20, KOH 10, NaCl 8, KOH-EGTA 0.2, ATP-Mg 2, GTPNa 0.3, pH 7.2 adjusted with KOH (1M), 290-300 mOsm.

Whole-cell access was established following formation of a gigaseal (>1 G Ω) between pipette tip and cell membrane^(b). Cells were voltage-clamped at $V_h = -70$ mV (with a EPC-7 amplifier, List Biologic, Campbell, CA), digitized at 10kHz using a Digidata 1322A board and data acquired through Clampex software version 10.2 (Molecular devices, Sunnyvale, CA, USA). Offset potentials were nulled directly before giga-seal formation. Immediately after establishing whole-cell access, the membrane potential of the neurons was routinely determined at the beginning of each experiment as well as firing patterns, measured in current-clamp mode, to confirm CA1 pyramidal cell identity. Junction potentials and voltage errors caused by small changes in series resistance were not corrected, but series resistance was regularly monitored throughout each experiment with a -5mV, 50ms pulse, and cells with more than 20% variation over time were excluded from the data. Holding current was also monitored and recordings were rejected if the holding current was greater than - 100 pA or if it abruptly changed.

Notes

2- Quality of giga-seal is fundamental for quality and stability of electrophysiological recordings

GigaOhm seal obtainment: Positive pressure avoids the tip of the pipette to acquire debris^(c). When the tip of the pipette is in close contact with the cell membrane, a 'dimple' is seen: it results from the physical contact between the pipette and the membrane, which is optically visible with a lighter reflection. Simultaneously, the pipette resistance increases due the blockade of electrical flow by the cell membrane. To obtain the gigaohm seal, negative pressure is applied and a resistance superior of 1 G Ω is rapidly achieved. To enter in whole-cell configuration, a fast strong pulse of negative pressure is applied, allowing patched membrane rupture and direct contact between pipette and cell content^(b).

(a) Pipette resistance correlates inversely with tip opening diameter, which can not be too small (easy to seal, but difficult to obtain whole-cell configuration) nor too large (difficult to seal, but easy to rupture cell membrane).

- (b) Internal solution is in direct contact with cell content in whole-cell configuration. Over time, the internal solution replaces the cell content (phenomenon known as “cell content dialysis” since the internal solution volume is greater than cell volume). It is crucial that the internal solution is electro-chemically similar to the cell content and allows for desired recordings.
- (c) Pipette tip ‘cleanliness’ is fundamental to assure a full seal between pipette tip and membrane, without cellular debris in between.

Electrically-evoked excitatory postsynaptic currents (eEPSCs)

Afferent-evoked excitatory postsynaptic currents (EPSCs) were elicited by 0.1 ms rectangular pulses (0.1 – 0.5 mA), delivered once every 15 s through a bipolar concentric wire electrode manually fabricated from platinum/iridium wire (25 μ m diameter, <800 k Ω impedance (Advent Research Materials) positioned either in the Schaffer collaterals afferents or Perforant Pathway. EPSCs were recorded from pyramidal cells from CA1 area, voltage-clamped at $V_h = -70$ mV and perfused with aCSF containing picrotoxin (PTX, GABA_AR antagonist, 50 μ M) to block the fast component of GABAergic transmission. eEPSC recordings were started not before the first 5–10 minutes after break-in to enable diffusion of intracellular solution in the soma and proximal dendrites. After 10 minutes of eEPSC amplitude stability, tested drugs were applied in the perfusion system (CCPA 30 nM, MRS5474 120 nM, DPCPX 100 nM). Averages of four consecutive individual recordings were used to plot and analyse data and the 10 minutes period before bath application of the drug was compared to the 30-40 minutes following its application.

Firing patterns

Firing patterns of CA1 pyramidal cells were determined in current-clamp mode immediately after achieving whole-cell configuration by a series of hyperpolarizing and depolarizing steps of current injection (1 second duration). Apart from visual identification through morphology (pyramidal shape) and location (somata in layer stratum pyramidal), CA1 pyramidal cells are characterized by slow firing frequencies (≤ 5 Hz), longer action potentials (≥ 0.8 ms) and for featuring spike-frequency adaptation (Figure 16). Increased depolarizing steps of current injection increased firing frequency until maximum firing frequency was reached. From that moment on, inactivation of voltage-dependent Na⁺ channels prevented the firing of more than a few initial action potentials. The analyzed population of CA1 pyramidal cells presented resting membrane potential of -62.0 ± 5.8 mV (mean \pm SD) (n=93 cells), in accordance with the literature (-62.5 ± 4.0 mV, Henze and Buzsáki). Characterization of the model used, pyramidal cells from CA1 area of acute hippocampal slices is in accordance with the literature, validating the used model.

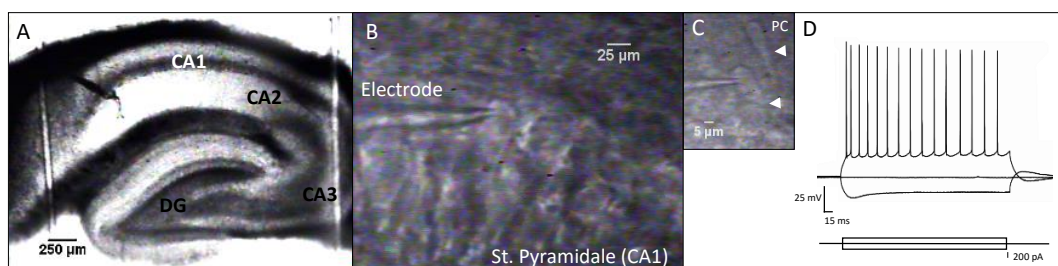


Figure 16. Localization, morphology and physiological properties of CA1 pyramidal cells in acute rat hippocampal slices. (A) Acute rat hippocampal slices exhibit defined cytoarchitectonic organization, with distinct structures, divided into Dentate Gyrus (DG) and Cornu Ammonis (CA) areas, subdivided in CA1, CA2 and CA3 areas, which are also subdivided in defined strata (or layers) (B) Stratum pyramidale (CA1) contains the cell bodies of pyramidal cells, the principal excitatory neurons of the hippocampus, disposed in a strata easily identified by the naked eye. (C) Pyramidal cells (PC) are morphologically characterized by a triangular shaped cell body (arrow) with a single axon and multiple basal dendrites. (D) Pyramidal cells are also identified functionally by their firing patterns in response to current injection through the recording electrode, as described in Methods. Note accommodation of firing frequency (time interval between 2 last action potentials (APs) is longer when compared to 2 first APs). Images and recordings in this figure were obtained for the purpose of this work.

Pharmacological tools

Isoflurane was from Abbot Laboratories (Barcelona, Spain). DPCPX (1,3-dipropyl-8-cyclopentylxanthine) (selective antagonist of A_1R), CCPA (selective agonist of A_1R), Picrotoxin ($GABA_A R$ antagonist) and MRS5474 were from Tocris Bioscience (Bristol, UK) and were prepared as a 5 mM stock solution in dimethylsulfoxide (DMSO). The percentage of DMSO in each experiment did not exceed 0.01%. Stock solutions were aliquoted and stored at -20°C until use. Dilutions of these stock solutions to the final concentration were made freshly before each experiment.

3. Data analysis

Features from eEPSCs recordings and firing patterns were extracted with Clampfit (pClamp 9.2 software, Axon) off-line. Graphical plotting and statistical analysis were conducted with the Prism Version 6.0 for Windows (GraphPad Software).

CA1 pyramidal cell images

Images were obtained with QuickStarter Studio v.8 program and edited with ImageJ (version 1.42q; NIH, USA).

eEPSC recordings

As previously explained, series resistance was monitored on-line and assessed off-line as a parameter of experience validity (if $>20\%$ of variation was observed, the experience was rejected). Amplitude of eEPSC, Holding current and Input Resistance (referred to as Membrane Resistance in Results) were measured as detailed in Table 11 and described in Figure 17 as parameters of interest. Membrane potential variation due to applied drug

was estimated, based on Ohm's Law (for more details, see Figure S2). In each experiment of eEPSC recording, averages of four consecutive individual recordings were used to plot time courses. Values refer to mean peak amplitude of eEPSCs and are represented as mean \pm Standard Error of the Mean (SEM) from n experiments. Effect of tested drug was analyzed comparing baseline (10 minutes before drug application) with tested drug (30-40 minutes after drug application). Statistical significance was assessed by a two-tailed Paired t test for the experimental versus control condition. A p value of 0.05 or less was considered to account for significant differences.

Firing Patterns

Firing patterns were elicited before each eEPSC recording as an additional mean of confirming CA1 pyramidal cell identity, as previously explained. A set of experiments were performed in which the goal was to obtain firing patterns as the final readout. To analyze the influence of the tested drug (MRS5474 120 nM) on intrinsic excitability parameters, resting membrane potential (RMP) and AP threshold were first analyzed and its difference calculated (ΔV_m). This set of experiments arose from the increased excitability observed in eEPSC recordings, in which AP firing was observed in 7 of 13 cells, from which only 6 cells maintained series resistance $< 20\%$ variation. Other parameters were also analyzed, namely AP Amplitude, AP rising time, AP decay time, fAHP, mAHP, sAHP, firing frequency, accommodation index and rheobase. In Table 11 and Figure 17 a description of how the measurement was performed. All these parameters have in their biological basis ionic channels and they constitute a target of for neuronal excitability, since a small alteration in these channels/parameters has implications in microcircuit communication.

RMP and AP Threshold: Cells with RMP > -50 mV were rejected. Outliers were identified as being $>2x$ Standard Deviation of the population for that parameter. Cells that were identified as outliers in one parameter were usually also outliers for other parameters and, regardless of that, were excluded from data set for all parameters. A pool of cells with maximal effect of MRS5474 (120 nM) at 1-2.5 hours after drug exposure was selected to analyze the effect of this drug in other parameters. To ensure the comparison between test treatment and control cells is valid, control cells were pooled so as the exposure to Picrotoxin and recovery period are within the same interval between control and test cells. Two tailed Unpaired t-test with Welch's Correction was used to statistically analyze differences, except for the paired design experiment, where two-tailed Paired t-test was used.

Firing Frequency: one-way ANOVA followed by Bonferroni's Multiple Comparison test was used.

sAHP: as the amplitude of the slow afterhyperpolarization following a train of action potentials correlated with the number of action potentials, firing patterns were pooled that responded with a spiking frequency of 16–20 Hz to a depolarizing current injection lasting 1 s.

Table 11. Parameter Analysis

Feature		Measure	Relevance
A. eEPSC Recordings			
Holding Current (pA) ^a		Mean of Holding current (first 150 ms)	Correlates with Membrane potential
Amplitude (pA) ^b		Difference between Peak Amplitude and Holding Current, after electrical stimulation	Measure of level of synaptic transmission
Input Resistance (MΩ) ^a		Derived from mean amplitude current in response to the test pulses according to Ohm's law (no capacitive currents flowing)	Correlates with specific membrane resistance ($R_{input}=R_m/A$). Reflects ion channels composition and functioning of the membrane
Series Resistance (MΩ)		Derived from peak amplitude current in response to the test pulses according to Ohm's law	Reflects the quality of the gigaseal. Parameter for experience validity
Time of Peak (ms) ^b		Difference between time of Peak Amplitude and time of Stimulus artifact	Reflects ion channels recruited in synaptic transmission
Membrane Potential (mV)		Estimate based on Ohm's Law: using holding current and membrane resistance, voltage is predicted	Estimate to measure the effect of drugs in the level of cell excitability.
B. Firing Pattern			
RMP (mV)		Measured right after whole-cell break-in	Intrinsic excitability properties
AP Threshold (mV)		Membrane potential at which its slope is >10 mV/ms in first AP fired	
AP Amplitude (mV)		Difference between AP Peak Amplitude and AP Threshold	
AP Rising time (ms)		Interval of time between AP Threshold and AP Peak Amplitude	
AP Decaying time (ms)		Interval of time between AP Peak Amplitude and relative minimum of fAHP	
fAHP (mV)		Difference between first relative minimum after AP (typically 1-5 ms) and AP Threshold	
mAHP (mV)		Difference between second relative minimum after AP (typically 20-100 ms) and AP Threshold	
sAHP (mV)		Difference between AP Tail current negative going peak and mean membrane potential voltage (V _h) measured in the first 200 ms.	
Firing Frequency (Hz)		Number of action potentials fired in a step of depolarizing current (interval of time is 1 sec)	
Accommodation index		Interspike interval of last two APs divided by interspike interval of first two APs.	
Rheobase (pA)		Minimum Injected current necessary to evoke AP firing in an excitable cell.	

Notes: a – Mean holding current was measured avoiding miniature EPSC

b – If EPSC was polysynaptic, data refer to the first peak analyzed

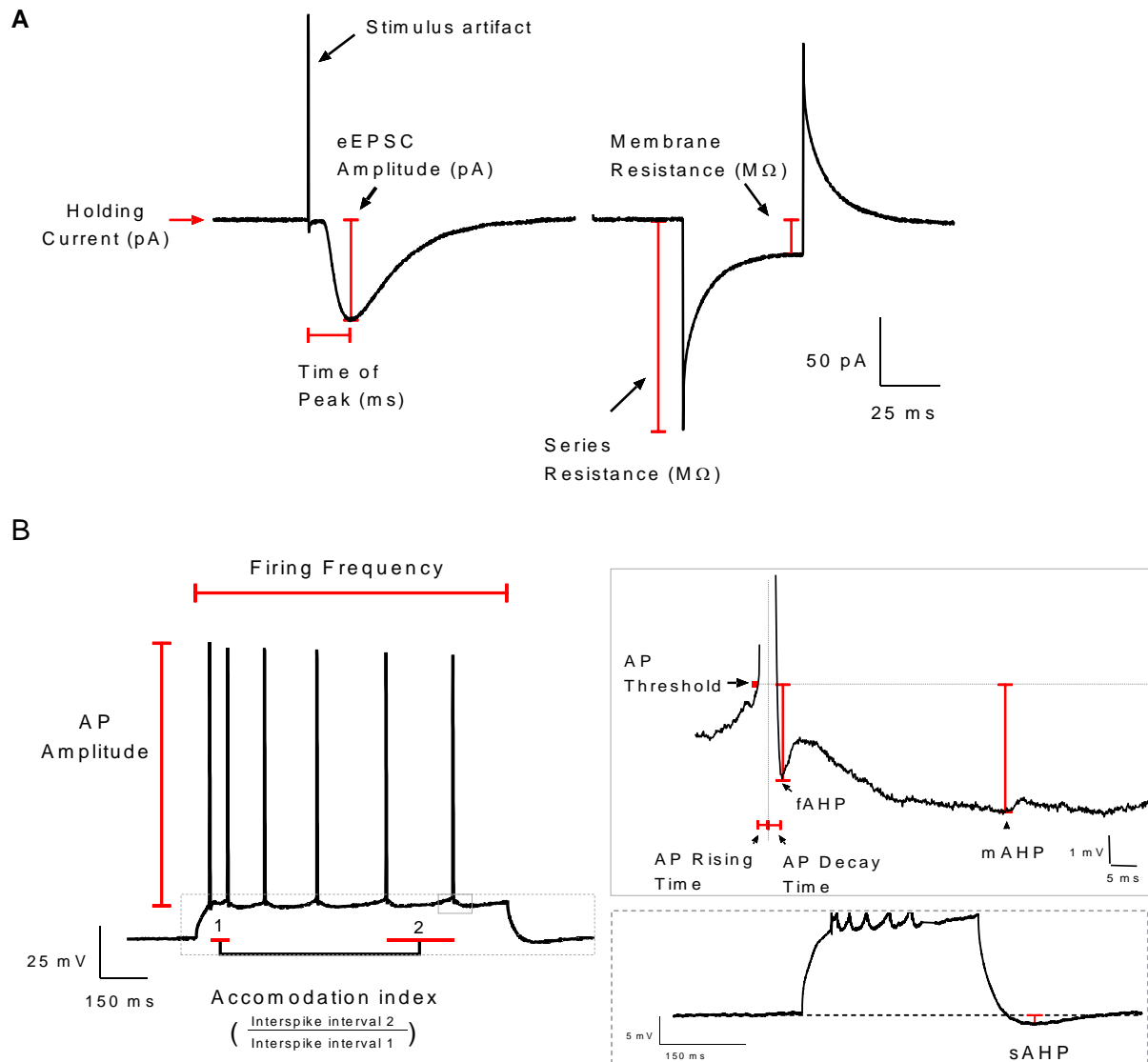


Figure 17. Illustrated parameter analysis. **A.** Example of a typical trace of eEPSC recording obtained. **B.** Example of a typical trace of a firing pattern recorded in CA1 pyramidal cells. Analyzed parameters are indicated with red lines to graphically illustrate how analysis was performed.

RESULTS

5. RESULTS

5.1. Activation of A₁ adenosine receptors (CCPA 30 nM) inhibits excitatory post-synaptic transmission of CA1 pyramidal cells of rat hippocampus.

Before testing the effect of MRS5474, it is important to test the consequences of A₁R activation effect on excitatory synaptic transmission as a proof of concept control, despite its broad description in the literature. To do so, an A₁R selective agonist was used, CCPA, at a concentration of 30 nM (100 fold superior to its K_i (0.4 nM)). CCPA was applied in the bath of acute hippocampal slices of wt rat and CA1 pyramidal cell excitatory synaptic transmission recorded via patch-clamp (whole-cell) technique. CA1 pyramidal cells were electrically stimulated at Perforant Path or Schaffer Colateral fibers that project onto CA1 pyramidal neurons. Since both are glutamatergic cells, excitatory-excitatory synapses were studied (with GABAergic synaptic transmission blocked by picrotoxin (50 μ M)).

5.1.1. CCPA (30 nM) decreases excitatory post-synaptic transmission

CCPA (30 nM) application gradually inhibits EPSC amplitude upon 2-3 minutes after application, which is concordant with CCPA concentration in the perfusion bath. At 20 minutes, maximum effect is reached and maintained. At 30-40 minutes, CCPA maximum effect was measured and it inhibited excitatory post-synaptic transmission by 70 ± 5.2 % (n=4, p=0.0009) (Figure 18). These observations are in accordance with the literature (inhibition of EPSC: 66 ± 3.0 % (n=8) Hargus et al, 2009).

CCPA application did not seem to change the kinetic properties of the EPSC (Figure 18.C.Inset) (assessed in monosynaptic signal, Figure S1) nor did it significantly change time of peak (12 ± 1.5 ms, n=4, p=0.1897) (Figure S1. E).

At the end of each experience with CCPA (30 nM), selective antagonists of AMPA/Kainate (CNQX, 10 μ M) and NMDA (DL-AP5, 50 μ M) receptors should have been applied to confirm the EPSC was only due to glutamatergic synaptic transmission. However, it has been widely described in the literature that blockade of GABA_AR by picrotoxin mainly inhibits GABAergic transmission and only glutamatergic synaptic transmission occurs (e.g. Jones and Kauer, 1999).

5.1.2. CCPA (30 nM) hyperpolarizes membrane potential of post-synaptic CA1 pyramidal cells of rat hippocampus

Also in line with a decreased excitatory synaptic transmission, CCPA hyperpolarizes membrane potential, which reduces the probability of action potential firing by the cell. Hyperpolarization was directly measured by injected current and CCPA application was observed to cause a 29 ± 4.5 pA shift from original injected current (n=3, *p=0.0226)

(Figure 19). The time course of the changes in this parameter was very similar to the time course of amplitude change, reinforcing the idea that it is an effect of CCPA application. It is, however, more relevant to know, in mV, the degree of this hyperpolarization. It represents a hyperpolarization of 6.6 ± 0.55 mV ($n=3$, $p=0.0075$) (Figure 20.), in accordance with calculations using a model for membrane potential prediction based on Ohm's Law, which was then validated (Figure S2).

From the entire set of experiences of CCPA application ($n=4$), one was performed with a complemented intracellular solution that contained QX-314 (5 mM), a voltage-dependent sodium channel blocker that prevented the firing of action potentials. This experience was relevant in the context of the following experiences with MRS5474, therefore it will be analyzed further on. The important feature that matters to retain is that QX-314 (5 mM) presence did not impair CCPA effect on EPSC amplitude, even though it significantly changed injected current and membrane resistance from whole-cell onset independently from CCPA application in the perfusion system. For that reason, we did not include these results in hyperpolarization or membrane resistance analysis.

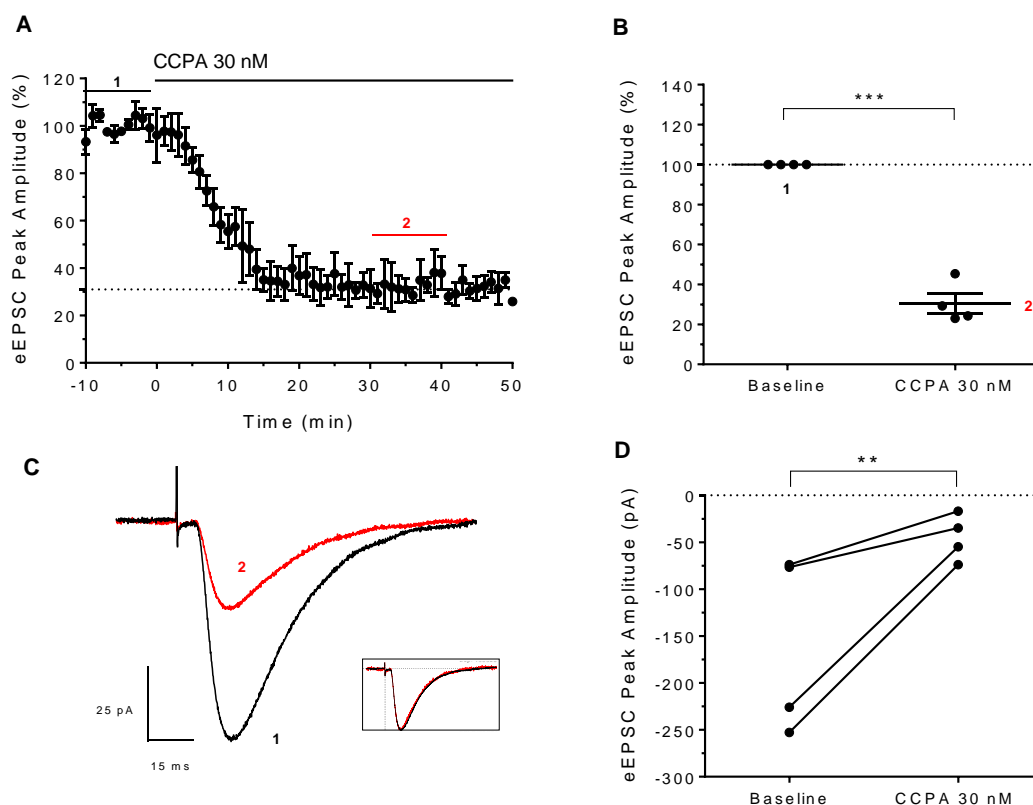


Figure 18. Activation of A1 adenosine receptors (by CCPA 30 nM) inhibits excitatory post-synaptic transmission of CA1 pyramidal cells of rat hippocampus. (A) Normalized averaged time-course changes in EPSC peak amplitude caused by superfusion of the selective A₁R agonist CCPA (30 nM) (applied as indicated by the horizontal bar). Each point represents the average of 4 individual responses, elicited once every 15 seconds. Data is presented as mean \pm SEM. **(C)** Representative EPSC averaged traces from one cell in (1) baseline (10 minutes before drug application) and (2) after CCPA application (30-40 minutes after drug application). Inset: Normalized

EPSC averaged traces. **(B)** Averages of normalized EPSC peak amplitude of (1) baseline and (2) after CCPA application. **(D)** EPSC amplitude (pA) of all cells in (A), in baseline (1) and after CCPA application (2). Values from each cell are connected with line. Two tailed Paired t-test was used for statistical analysis of CCPA effect (**p-value < 0.01; ***p-value < 0.001). n=4 cells, from 4 animals.

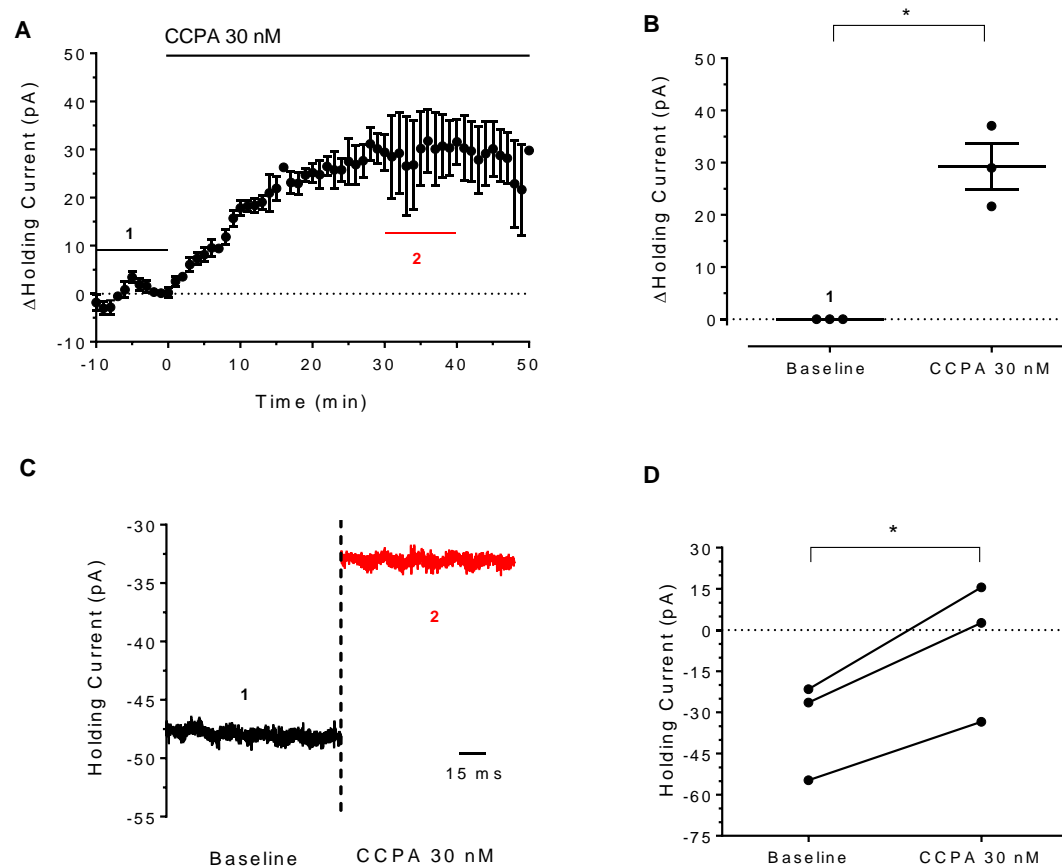


Figure 19. Activation of A1 adenosine receptors (CCPA 30 nM) hyperpolarizes membrane potential of post-synaptic CA1 pyramidal cells of rat hippocampus. (A) Averaged time-course changes in Holding current (pA) - necessary to maintain whole-cell voltage-clamp recordings ($V_h = -70$ mV) from CA1 pyramidal cells – caused by superfusion of the selective A₁R agonist CCPA (30 nM) (applied as indicated by the horizontal bar). Each point represents the average of 4 individual responses, measured every 15 seconds. Data is presented as mean \pm SEM. **(C)** Representative EPSC averaged traces from one cell in (1) baseline (10 minutes before drug application) and (2) after CCPA application (30-40 minutes after drug application). **(B)** Averages of absolute change in Holding current of (1) baseline and (2) after CCPA application. **(D)** Holding current (pA) of all cells in (A), in baseline (1) and after CCPA application (2). Values from each cell are connected with line. Paired t-test was used for statistical analysis of CCPA effect (*p-value < 0.05). n=3 cells, from 3 animals. All recorded cells had resting membrane potential more positive than -70 mV, around -66 mV. Upon CCPA application, it is necessary to inject less current to maintain the membrane potential at -70 mV. So, the membrane potential hyperpolarized.

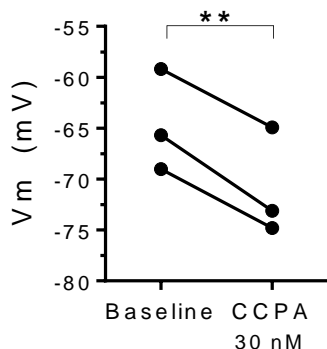


Figure 20. Predicted post-synaptic hyperpolarization effect of A₁R activation by CCPA (30 nM). Physiologically relevant measure of CCPA (30 nM) hyperpolarization of membrane potential. Ohm's Law is applied (ΔI corresponds to change in holding current; ΔR corresponds to total resistance approximation ($R_m + R_a + R_p$). Model validation in Figure S2. Values from each cell are connected with line. Paired t-test was used for statistical analysis of CCPA effect (**p-value < 0.01). n=3 cells, from 3 animals. R_m : Membrane Resistance; R_a : Series Resistance; R_p : Pipette Resistance.

5.1.3. CCPA (30 nM) decreases Membrane Resistance

CCPA (30 nM) application significantly decreased membrane resistance, by $9.9 \pm 1.3\%$, (n=3, p=0.05) (Figure 21.). This has already been observed by others (Bannon et al, 2014) and results from K⁺ channel opening responsible for membrane potential hyperpolarization. According to Ohm's Law, a decrease in membrane resistance would lead to an increased in the amplitude of EPSC. Since CCPA application inhibits the eEPSC amplitude, this inhibition can not be due to decreased resistance membrane.

In this set of experiments, the effects observed in all experiences performed were reproducible, followed the same time course changes and presented relatively low dispersion of values, as assessed by Standard deviation. This is in accordance with a robust widely described effect of A₁R activation.

Confounding variables

Confounding variables that may explain the decreased amplitude of EPSC in the presence of CCPA (30 nM) were studied, namely age, perfusion system rate, pipette resistance, slice thickness, pipette resistance, gigaseal and series resistance (Table S1). Even though no correlation analysis is possible to be performed due to the small amount of experiments (n=4), none parameter seemed to correlate with the EPSC amplitude, except perfusion system rate. Diminished perfusion system rate related with a smaller effect of CCPA on EPSC amplitude, which is expected if the time of analysis (30-40 minutes after CCPA application) is fixed.

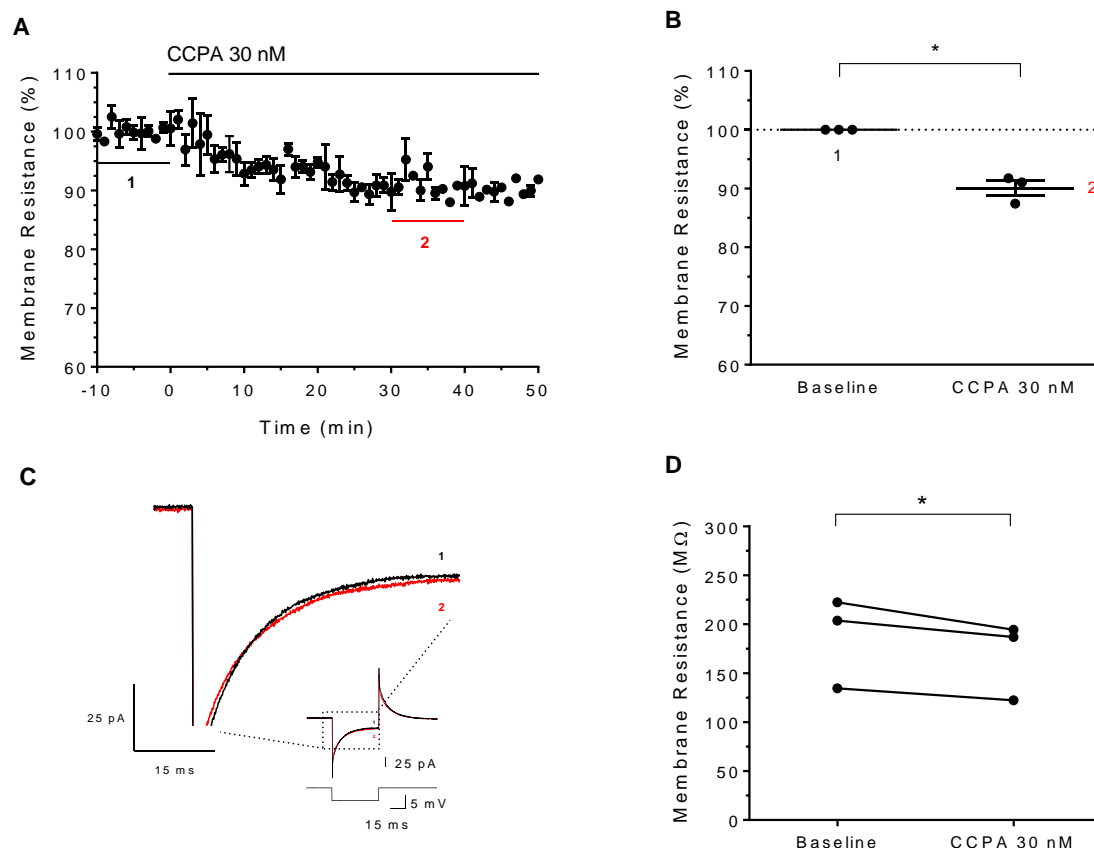


Figure 21. Activation of A₁ adenosine receptors (CCPA 30 nM) decreases membrane resistance of post-synaptic CA1 pyramidal cells of rat hippocampus. (A) Normalized averaged time-course changes in membrane resistance caused by superfusion of the selective A₁R agonist CCPA (30 nM) (applied as indicated by the horizontal bar). Each point represents the average of 4 individual responses, elicited once every 15 seconds. Data is presented as mean±SEM. (C) Representative averaged traces from quadratic pulse one cell in (1) baseline (10 minutes before drug application) and (2) after CCPA application (30-40 minutes after drug application). (B) Averages of normalized membrane resistance of (1) baseline and (2) after CCPA application. (D) Membrane Resistance (MΩ) of all cells in (A), in baseline (1) and after CCPA application (2). Values from each cell are connected with line. Two tailed Paired t-test was used for statistical analysis of CCPA effect (*p-value < 0.05). n=3 cells, from 3 animals.

5.1.4. Inhibitory effect of CCPA (30 nM) on excitatory synaptic transmission is A₁R-dependent

To confirm that the inhibitory effect of CCPA in EPSC is an A₁R mediated effect, we tested if the effect of CCPA (30 nM) is abolished in the presence of an adenosine A₁R antagonist (DPCPX, 100 nM). The presence of DPCPX (100 nM) reversed the inhibitory effect of CCPA (30 nM) and even blocked the inhibitory tonus of A₁R, by increasing EPSC amplitude and even led to action potential firing (Figure 22.).

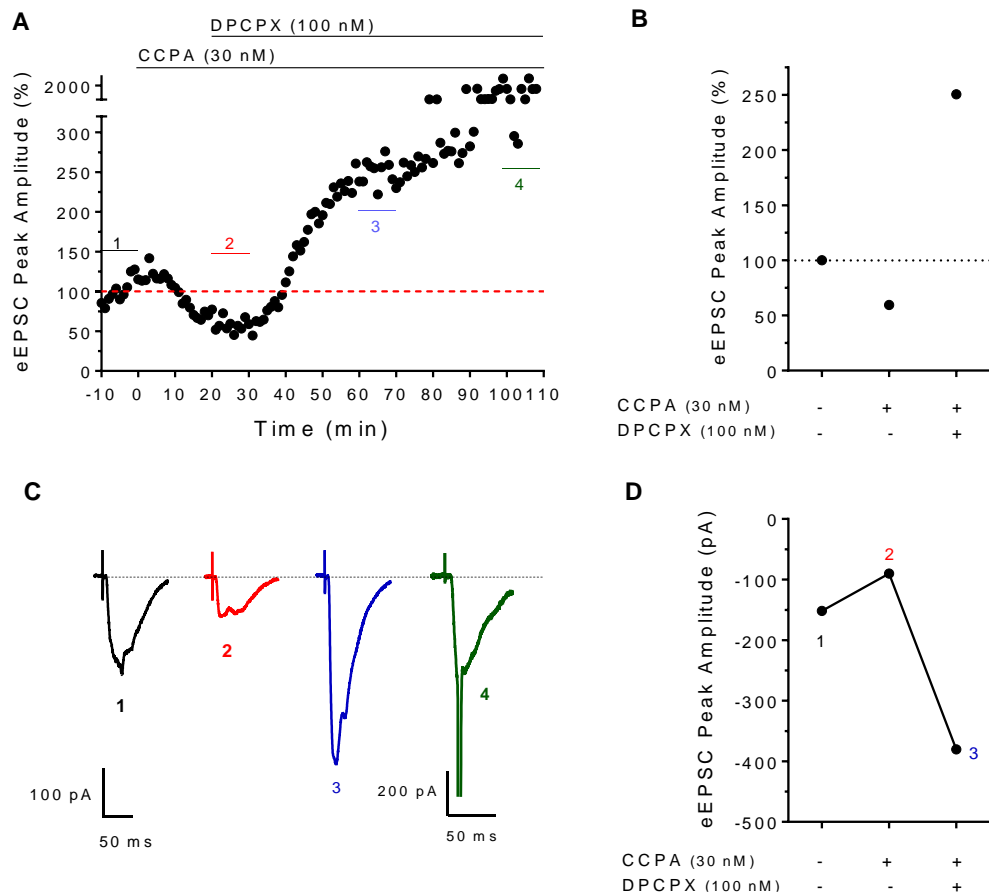


Figure 22. Inhibitory effect of CCPA (30 nM) on excitatory synaptic transmission is A₁R-dependent. (A) Averaged time-course changes in EPSC peak amplitude caused by superfusion of the selective A₁R agonist CCPA (30 nM) and A₁R antagonist DPCPX (100 nM) (applied as indicated by the horizontal bars). Each point represents the average of 4 individual responses, elicited once every 15 seconds and data is presented as mean±SEM. Representative averaged traces are shown in (C), with number 1 representing EPSC of baseline (10 minutes before drug application), number 2 representing EPSC after CCPA application (30-40 minutes after drug application), number 3 representing EPSC after DPCPX + CCPA application (60-70 minutes) and number 4 an action potential fired. To compare the effect of CCPA, averages of EPSC peak amplitude of baseline (10 minutes before drug application) and after CCPA application (30-40 minutes after drug application) are plotted in (B and D). Each one relative to (A) and (B), respectively. n=1 cell.

The number of experiences is too low, only $n=1$, for which more experiences should have been performed. However, this set of experiments is a standard A_1R activation control which has been described in the literature, by Wu et al (1994) in CA1 neurons and Hargus et al (2009) in subiculum pyramidal neurons, in the hippocampal slice model, for example.

The isolated effect of DPCPX (100 nM) on eEPSC peak amplitude should have been tested as a control. However, there are considerable observations in the literature for A_1R antagonism leading to increased synaptic transmission, either by increasing extracellular calcium concentration in the synaptic and pyramidal cell soma layer (CA1 of rat hippocampal slices) (Schubert, 1988) or increasing synaptic transmission in fEPSC (Diógenes *et al.*, 2014).

This chapter provided the necessary control for A_1R activation outcomes, which are in accordance with the literature. Being so, not only a classical activation of A_1R is herein described and validated but also the operator ability to perform this technique was demonstrated.

DISCUSSION & FUTURE WORK

6. DISCUSSION AND FUTURE WORK

Adenosine acts as an endogenous anticonvulsant (During and Spencer, 1992) (Figure 35) that was shown to protect against pharmacoresistant epilepsy (Gouder et al, 2013). However its systemic administration also triggers unacceptable side effects, mainly cardiovascular (Monopoli et al, 1994; Gouder et al, 2013), that prevent its use as an AED.

A new adenosine derivative MRS5474 was shown to protect against elicited seizures in the pharmacoresistant epilepsy model 6Hz *in vivo* without observable behavioral toxicity (up to 30 mg/kg), unlike the classical selective A₁R agonist CCPA (Tosh et al, 2012).

In this work, we aimed at determining the synaptic target of MRS5474, which could help to shift the search for new AEDs towards a novel target. Since MRS5474 was designed and shown to present high A₁R affinity and full agonism with moderate selectivity, we hypothesized that MRS5474 decreases excitatory synaptic transmission through A₁R activation in CA1 pyramidal cells of the hippocampus.

6.1. CCPA is a classical A₁R agonist

Before testing MRS5474 (120 nM), we studied A₁R activation by CCPA (30 nM). CCPA inhibited excitatory synaptic transmission by decreasing EPSC amplitude and membrane potential hyperpolarization. This effect was reversed in the presence of an A₁R antagonist (DPCPX, 100nM), even though the number of experiences was too low (n=1). These results are already extensively described in the literature (Bannon et al, 2014; Heidarianpour et al, 2006, Dunwiddie and Fredholm, 1989), and the main objective was to test if in our experimental conditions we could detect A₁R induced inhibition action of an A₁R agonist.

A₁R activation by CCPA explains both its anticonvulsant properties as well as the behavioral toxicity observed *in vivo* in the pharmacoresistant model of epilepsy (6 Hz) (Tosh et al, 2012). Since A₁R are also present in the heart, A₁R activation leads to suppressed automaticity of cardiac pacemakers and inhibition of AV-nodal conduction, resulting in decreased heart rate and blood pressure (Schindler et al, 2005; Mustafa et al, 2009). These effects, even though not directly measured in Tosh et al. work (2012), may explain the inability of mice to stay in the rotarod platform during 1 minute without falling more than 3 times at a CCPA dose of 0.84 mg/kg (when ED₅₀^(CCPA)= 0.12 mg/kg).

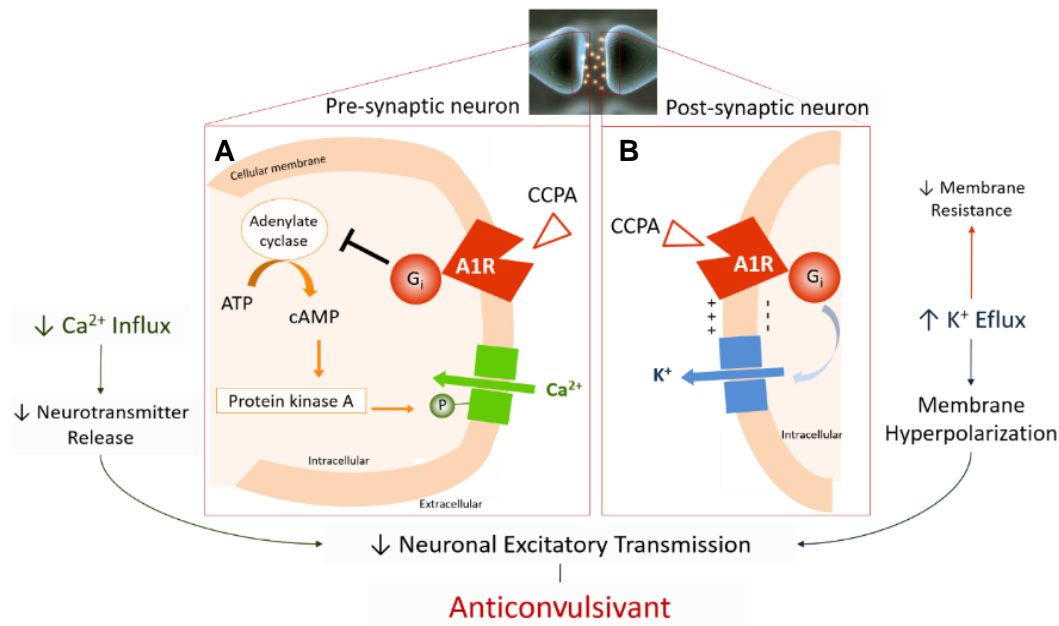


Figure 35. Anticonvulsant mechanisms of A₁R activation. **(A)** Pre-synaptic A₁R activation results in lower Ca²⁺ influx, preventing neurotransmitter vesicles to dock and release its content to the synaptic cleft in a synchronized way. This process is described in the literature to be mediated by inhibitory G-protein activation that inhibits adenylate cyclase activity. This results in a lower production of cAMP from ATP, necessary to activate protein kinase A. Upon activation, protein kinase A phosphorylates Ca²⁺ channels, allowing its entrance into the intracellular environment. Therefore, lower cAMP concentration diminishes, through protein kinase A, the amount of phosphorylated Ca²⁺ channels, diminishing the entrance of Ca²⁺ to the cell. Since Ca²⁺ is necessary for neurotransmitter vesicle docking in the pre-synaptic membrane and posterior exocytosis of neurotransmitter content, lower concentrations of Ca²⁺ lead to decreased synchrony of neurotransmitter release, which is reflected in a lower amplitude EPSC. **(B)** Post-synaptic A₁R activation explains membrane potential hyperpolarization (Bannon et al, 2014), since A₁R are coupled to inhibitory G-protein which directly activate inward-rectifier potassium (GIRK) channels (Marck and Herlitze, 2000). It leads to K⁺ efflux, increasing the difference between membrane potential. Thus, the membrane potential is hyperpolarized. At the same time, opening of K⁺ channels decreases membrane resistance, which has also been observed. Both decreased neurotransmitter release synchrony and membrane potential hyperpolarization lead to decreased excitatory synaptic transmission, which contributes to a lower probability of seizure generation onset and propagation.

7. CONCLUSION

Being able to use the adenosinergic system as a powerful anticonvulsivant mechanism for pharmacoresistant epilepsy opens the way to a strategy of treatment that had unfortunately been closed due to its severe side effects.

MRS5474 is a new hope, since it is a new adenosine derivative that protects against elicited seizures in the pharmacoresistant model of epilepsy 6 Hz *in vivo* without observable side effects, unlike the classical selective A₁R agonist CCPA.

Searching for its synaptic target and mechanism of action, we have shown that MRS5474 is not a classical A₁R agonist, since it does not decrease excitatory synaptic transmission in CA1 pyramidal neurons of healthy rat hippocampus, as occurs with selective A₁R activation with CCPA. In spite of being unexpected, since MRS5474 was shown to have high affinity to A₁R in overexpressing cells, it opens the way for a different mechanism of action that may explain the apparent absence of classical A₁R side effects upon *in vivo* administration.

In fact, we have also shown that MRS5474 presents a pro-excitatory role in intrinsic excitability of CA1 pyramidal cells in healthy rats, when studying excitatory synaptic transmission. RMP depolarization, increased firing frequency and decreased amplitude of AHP currents (fAHP and mAHP) support an excitatory role of MRS5474, which, by itself, is not able to explain the anticonvulsivant properties of MRS5474. However, it points towards a selective and complex mechanism of action, that can only be fully understood with a wider knowledge of MRS5474 effect on other system components, including inhibitory synaptic transmission of interneurons and CA1 pyramidal cells in healthy and in epileptic tissue.

If the increased intrinsic excitability caused by MRS5474 on CA1 pyramidal cells is also observed at a greater extent in interneurons, then the global net effect of MRS5474 would be inhibitory. This hypothesis could explain not only its anticonvulsivant properties but also the lack of observable side effects. Unveiling the synaptic target of MRS5474 and its mechanism of action is, thus, an interesting track to bypass the negative implications of using the adenosinergic system and take advantage of its powerful anticonvulsivant properties against pharmacoresistant epilepsy.

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